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FORM PTO-1390 (REV. 11-94) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)		8484-089-999 09/674794
INTERNATIONAL APPLICATION NO. PCT/DE99/01350	INTERNATIONAL FILING DATE 5 May 1999	PRIORITY DATE CLAIMED 5 May 1998

TITLE OF INVENTION

MULTIVALENT ANTIBODY CONSTRUCTS

APPLICANT(S) FOR DO/EO/US
Little *et al.*

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

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6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application in response to the Written Opinion
   a. ☒ are transmitted herewith.
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☒ A translation of the amendments to the claims in Response to the Written Opinion.

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (unexecuted).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

    First page of published PCT Application under no. WO99/57150;
    Request for International Application;
    International Search Report;
    Request for Preliminary Examination;
    Written Opinion;
    Response to Written Opinion and English translation of claims as amended;
    Marked-up copy of the Substitute Specification; and
    Return Post Card.

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INTERNATIONAL FILING DATE
5 May 1999

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17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	27 - 20	7	X $ 18.00	$ 126.00
INDEPENDENT CLAIMS	1 - 3	0	X $ 80.00	0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 270.00	$ 270.00
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY** ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) .... $ 690 ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .... $ 710 ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... $1000 ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) .... $ 100 ☒ Filing with EPO or JPO search report .... $ 860				$ 860.00
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				
TOTAL OF ABOVE CALCULATIONS				= 1,256.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).				- $ 0.00
SUBTOTAL				= 1,256.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				+
TOTAL FEES ENCLOSED				$ 1,256.00

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a. ☐ A check in the amount of $_ to cover the above fees is enclosed.
b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $_ to cover the above fees. A copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☒ Other instructions
Please enter the Preliminary Amendment prior to counting the claims for determination of the fee, and prior to examination.

19. ☒ All correspondence for this application should be mailed to
PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

20. ☒ All telephone inquiries should be made to (212) 790-2803

Birgit Millauer
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SIGNATURE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Little *et al.*

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: Herewith

Examiner: To be assigned

For: **MULTIVALENT ANTIBODY CONSTRUCTS**

Attorney Docket No.: 8484-089-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with Rule 111 of the Rules of Practice, 37 C.F.R. § 1.111, please consider and enter the following amendments and remarks.

AMENDMENTS

IN THE SPECIFICATION:

Please replace the specification as filed in PCT/DE99/01350 by the enclosed Substitute Specification under 37 C.F.R. § 1.125. The Substitute Specification has been prepared solely for the purpose of complying with the rules of practice; it does not introduce new matter. A marked-up copy of the Substitute Specification showing any matter being added and any matter being deleted from the original specification is enclosed in accordance with 37 C.F.R. § 1.125(b)(2).

IN THE CLAIMS:

Please amend the claims as follows:

1. (Amended) A multivalent F_v antibody construct having at least four variable domains [which], wherein said vaiable domains are linked with one another via [the] a peptide [linkers] linker 1, a peptide linker 2 and a peptide linker 3, wherein [the] said peptide [linkers] linker 1 and said peptide linker 3 have [0] about 1 to about 10 amino acids.

2. (Amended) The F_v antibody construct [according to claim] of Claim 1, wherein [the] said peptide [linkers] linker 1 and peptide linker 3 have the amino acid sequence GG.

3. (Amended) The F_v antibody construct [according to claim] of Claim 1 [or 2], wherein [the] said F_v antibody construct is bivalent.

4. (Amended) The F_v antibody construct [according to claim] of Claim 3, wherein [the] said peptide linker 2 has about 11 to about 20 amino acids.

5. (Amended) The F_v antibody construct [according to claim] of Claim 3 or 4, wherein [the] said peptide linker 2 has the amino acid sequence $(G_4S)_4$.

6. (Amended) The F_v antibody construct [according to claim] of Claim 1 [or 2], wherein [the] said F_v antibody construct is tetravalent.

7. (Amended) The F_v antibody construct [according to claim] of Claim 6, wherein [the] said peptide linker 2 has about 3 to about 10 amino acids.

8. (Amended) The F_v antibody construct [according to claim] of Claim 6 or 7, wherein [the] said peptide linker 2 comprises the amino acid sequence GGPGS.

9. (Amended) The F_v antibody construct [according to any of claims] of Claim 1 [to 8], wherein [the] said F_v antibody construct is multispecific.

10. (Amended) The F_v antibody construct [according to claim] of Claim 9, wherein [the] said F_v antibody construct is bispecific.

11. (Amended) The F_v antibody construct [according to any of claims] of Claim 1 [to 8], wherein [the] said F_v antibody construct is monospecific.

12. (Amended) A method of producing the multivalent F_v antibody construct [according to any of claims] of Claim 1 [to 11, wherein DNAs coding for the], comprising:

(a) ligating nucleic acids encoding a peptide [linkers] linker 1, a peptide linker 2 and a peptide linker 3 [are ligated] with [DNAs coding for the] nucleic acids encoding four variable domains of an F_v antibody construct such that [the] said peptide [linkers] linker 1, 2, and 3 link the variable domains with one another; and

(b) subcloning the [resulting DNA molecule is expressed in] nucleic acid of step (a) into an expression plasmid.

13. (Amended) [Expression] An expression plasmid [coding for the multivalent F_v antibody construct according to any of claims 1 to 11] comprising the nucleic acid of Claim 22.

14. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pDISC3x19-LL as deposited with DSM.

15. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pDISC3x19-SL as deposited with DSM.

16. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pPIC-DISC-LL as deposited with DSM.

17. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pPIC-DISC-SL as deposited with DSM.

18. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pDISC5-LL as deposited with DSM.

19. (Amended) The expression plasmid [according to claim] of Claim 13, [namely] wherein said expression plasmid is pDISC5-SL as deposited with DSM.

20. (Amended) [Use of] A composition comprising the multivalent F_v antibody construct [according to any] of [claims] Claim 1 [to 11] for [the] diagnosis and/or treatment of [diseases] a disease.

21. (Amended) [Use according to claim] The composition of Claim 20, wherein [the diseases are] said disease is a viral, a bacterial or a tumoral [diseases] disease.

Please add the following new Claims 22-25:

22. (New) A nucleic acid encoding the F_v antibody construct of Claim 1.

23. (New) A host cell comprising the expression plasmid of Claim 13.

24. (New) A method of treating a disease, comprising administering the composition of Claim 20.

25. (New) A method of making a multivalent F_v antibody construct, comprising cultivating the host cell of Claim 23 under conditions that said multivalent F_v antibody construct is expressed.

REMARKS

The above amendments do not introduce new matter, and they are fully supported by the specification of the subject application and the claims as originally filed.

Applicants respectfully request that the above-made amendments be made of record in the file history of the instant application.

Respectfully submitted,

Date November 3, 2000

43,341

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MULTIVALENT ANTIBODY CONSTRUCTS

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MULTIVALENT ANTIBODY CONSTRUCTS

This is a national phase filing of the Application No. PCT/DE99/01350, which was filed with the Patent Corporation Treaty on May 5, 1999, and is entitled to priority of the German Patent Application 198 19 846.9, filed May 5, 1998.

I. FIELD OF THE INVENTION

The present invention relates to multivalent F_v antibody constructs, expression plasmids which code for them, and a method for producing the F_v antibody constructs as well as the use thereof.

II. BACKGROUND OF THE INVENTION

Natural antibodies are dimers and are therefore referred to as bivalent. They have four variable domains, namely two V_H domains and two V_L domains. The variable domains serve as binding sites for an antigen, a binding site being formed from a V_H domain and a V_L domain. Natural antibodies recognize one antigen each, so that they are also referred to as monospecific. Furthermore, they also have constant domains which add to the stability of the natural antibodies. On the other hand, they are also co-responsible for undesired immune responses which result when natural antibodies of various animal species are administered mutually.

In order to avoid such immune responses, antibodies are constructed which lack the constant domains. In particular, these are antibodies which only comprise the variable domains. Such antibodies are designated F_v antibody constructs. They are often available in the form of single-chain monomers paired with one another.

However, it showed that F_v antibody constructs only have little stability. Therefore, their usability for therapeutic purposes is strongly limited.

Thus, it is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

According to the invention this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a multivalent F_v antibody construct having at least four variable domains which are linked with each over via the peptide linkers 1, 2 and 3. The invention also concerns expression plasmids which code for such an F_v antibody construct and a method of producing the F_v antibody constructs as well as their use.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the genetic organization of an F_v antibody construct (A) according to the invention and schemes for forming a bivalent (B) or tetravalent F_v antibody construct (C). Ag: antigen; His_6: six C-terminal histidine residues; stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

FIGURE 2 shows the scheme for the construction of the plasmids pDISC3x19-LL and pDISC3x19-SL. c-myc: sequence coding for an epitope which is recognized by the antibody 9E1, His_6: sequence which codes for six C-terminal histidine residues; PelB: signal peptide sequence of the bacterial pectate lyase (PelB leader); rbs: ribosome binding site; Stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

FIGURE 3 shows a diagram of the expression plasmid pDISC3x19-LL. 6xHis: sequence which cods for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; ColE1: origin of the DNA replication; f1; Lac P/Of: wt lac-operon promoter/operator; linker 1: sequence which codes for a GlyGly dipeptide linking the V_H and V_L domains; linker 2: sequence coding for a $(Gly_4Ser)_4$ polypeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 4 shows a diagram of the expression plasmid pDISC3x19-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for ß-lactamase which is responsible for the ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope recognized by the 9E10 antibody; ColE1: origin of DNA replication; f1-IG: intergenic region of the bacteriophage f1; Lac P/Of: wt lacoperon promoter/operator: linker 1: sequence which codes for a GyGly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide which links the hybrid scFv fragments; Pe1-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 5 shows the nucleotide sequence and the amino acid sequence derived therefrom of the bivalent F_v antibody construct encoded by the expression plasmid pDIS3x19-LL. c-myc epitope: sequence coding for an epitope which is recognized by the antibody 9E10; CDR: region determining the complementarity; framework: framework region; His6 tail: sequence which codes for six C-terminal histidine residues; Pe1B leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 6 shows the nucleotide sequence and the derived amino acid sequence of the tetravalent F_v antibody construct encoded by the expression plasmid pDISC3x19-SL. c-myc epitope: sequence coding for an epitope which is recognized by the 9E10 antibody; CDR: region determining complementarity; framework: framework region; His6 tail: sequence coding for the six C-terminal histidine residues; Pe1B leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 7 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene which codes for an α-factor leader sequence and a gene coding for the tetravalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-SL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae-α* factor secretion signal; V_H: variable region of the heavy chain. Rhombs indicate the signal cleaving sites.

FIGURE 8 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene coding for an α-factor leader sequence and a gene which codes

for the bivalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-LL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae-α* factor secretion signal; V_H: variable region of the heavy chain. Rhombs show the signal cleaving sites.

FIGURE 9 shows a diagram of the expression plasmid pDISC5-LL. 6xHis: sequence coding for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilizing DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon-promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which codes for a GlyGly dipeptide connecting the V_H and V_L domains; linker 2: sequence which codes for a $(Gly_4Ser)_4$ polypeptide linking the hybrid scFv fragments; M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader; signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site which originates from the *E. coli* LacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; V_H and V_L: variable region of the heavy and light chains.

FIGURE 10 shows a diagram of the expression plasmid pDISC6-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs: c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilized DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which cods for a Glygly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide linking the hybrid scFv fragments: M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site originating from the *E. coli* lacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the

bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; V_H and V_L: variable region of the heavy and light chains.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

Therefore, the subject matter of the present invention relates to a multivalent F_v antibody construct which has great stability. Such a construct is suitable for diagnostic and therapeutic purposes.

The present invention is based on the applicant's insights that the stability of an F_v antibody construct can be increased if it is present in the form of a single-chain dimer where the four variable domains are linked with one another via three peptide linkers. The applicant also recognized that the F_v antibody construct folds with itself when the middle peptide linker has a length of about 10 to 30 amino acids. The applicant also recognized that the F_v antibody construct folds with other F_v antibody constructs when the middle peptide linker has a length of about up to 10 amino acids so as to obtain a multimeric, *i.e.*, multivalent, F_v antibody construct. The applicant also realized that the F_v antibody construct can be multispecific.

According to the invention the applicant's insights are utilized to provide a multivalent F_v antibody construct which comprises at least four variable domains which are linked with one another via peptide linkers 1, 2 and 3.

The expression "F_v antibody construct" refers to an antibody which has variable domains but no constant domains.

The expression "multivalent F_v antibody construct" refers to an F_V antibody which has several, but at least four, variable domains. This is achieved when the single-chain F_v antibody construct folds with itself so as to give four variable domains, or folds with other single-chain F_v antibody constructs. In the latter case, an F_v antibody construct is given which has 8, 12, 16, etc., variable domains. It is favorable for the F_v antibody construct to have four or eight variable domains, *i.e.*, it is bivalent or tetravalent (FIGURE 1). Furthermore, the variable domains may be equal or differ from one another, so that he

antibody construct recognizes one or several antigens. The antibody construct preferably recognizes one or two antigens, *i.e.*, it is monospecific and bispecific, respectively. Examples of such antigens are proteins CD19 and CD3.

The expression "peptide linkers 1, 3" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linkers 1 and 3 may be equal or differ from each other. Furthermore, the peptide linker may have a length of about 0 to 10 amino acids. In the former case, the peptide linker is only a peptide bond from the COOH residue of one of the variable domains and the NH_2 residue of another of the variable domains. The peptide linker preferably comprises the amino acid sequence GG.

The expression "peptide linker 2" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linker may also have a length of about 3 to 10 amino acids, in particular 5 amino acids, and most particularly the amino acid sequence GGPGS, which serves for achieving that the single-chain F_v antibody constructs. The peptide linker can also have a length of about 11 to 20 amino acids, in particular 15 to 20 amino acids, and most particularly the amino acid sequence $(G_4S)_4$, which serves for achieving that the single-chain F_v antibody construct folds with itself.

An F_v antibody constructs according to the invention can be produced by common methods. A method is favorable in which DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid. Reference is made to Example 1 to 6. As to the expressions "F_v antibody construct" and "peptide linker" reference is made to the above explanations and, by way of supplement, to Maniatis, T. *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

DNAs which code for an F_v antibody construct according to the invention also represent a subject matter of the present invention. Furthermore, expression plasmids which contain such DNAs also represent a subject matter of the present invention. Preferred

expression plasmids are pDISC3x19-LL, pDISC3x19-SL, pPIC-DISC-LL, pPIC-DISC-SL, pDISC5-LL and pDISC6-SL. The first four were deposited with the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellen) [German-type collection for micro-organisms and cells] on April 30, 1998 under DSM 12150, DSM 12149, DSM 12152 and DSM 12151, respectively.

Another subject matter of the present invention relates to a kit, comprising:

(a) an F_v antibody construct according to the invention, and/or

(b) an expression plasmid according to the invention, and

(c) conventional auxiliary agents, such as buffers, solvents and controls.

One or several representatives of the individual components may be present.

The present invention provides a multivalent F_v antibody construct where the variable domains are linked with one another via peptide linkers. Such an antibody construct distinguishes itself in that it contains no parts which can lead to undesired immune reactions. Furthermore, it has great stability. It also enables to bind several antigens simultaneously. Therefore, the F_v antibody construct according to the invention is perfectly adapted to be used not only for diagnostic but also for therapeutic purposes. Such purposes can be seen as regards any disease, in particular a viral, bacterial or tumoral disease.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Construction of the Plasmids Pdisc3x19-ll and Pdisc3x9-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Bacteria

The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov *et al.*, 1996, *J.-Immunol. Meth.* 196:51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov *et al.*, 1997, *Protein Eng.* 10:445-453), respectively, were used for the construction of expression plasmids for a single-chain F_v antibody construct. A PCR fragment 1 of the V_H domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-TTAGATCTATTAAAGAGGAGAAATTAACC, and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC (FIGURE. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR fragment 2 of the V_L domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidinyl tail, was produced using the primers DP3, 5;-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA, and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-GGTGATGTGAGTTTAGG. The PCR fragment 2 was cleaved by HindIII and XbaI and ligated with the HindIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 (FIGURE 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG and either Li-1, 5'-TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-CCACCACCGCTACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATC AGCCCG, for the production of a long flexible $(Gly_4Ser)_4$ inter-scFV linker (PCR fragment 3, FIGURE 2) or Li-2, 5'-TATATA-CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATCAGCCG, for the production of a short rigid GGPGS linker (PCR fragment 4, FIGURE 2). The expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the NcoI/PvuII restriction fragment from pHOG19-3, comprising the vector framework and the

NcoI/PVuII-cleaved PCR fragments 3 and 4, respectively (FIGURES 3 and 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F_v antibody constructs are indicated in FIGURES 5 and 6, respectively.

B. Example 2: Construction of the Plasmids Ppic-disc-ll and Ppic-disc-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Yeast

(A) Construction of pPIC-DISC-SL

The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal, followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli*. The gene which codes for the tetravalent F_v antibody construct (scDia-SL) was amplified by means of PCR by the template pDIC3x19-SL using the primers 5-PIC, 5'-CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG. The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences of the tetravalent F_v antibody construct are shown in FIGURE 7.

(B) Construction of pPIC-DISC-LL

The construction of pPIC-DISC-LL was carried out on the basis of pPICZαA (Invitrogen BV, Leek, Netherlands) and pDISC3x19-LL (FIGURE 3). The plasmid -DNA pPICZαA was cleaved by EcoRI. The overhanging 5'-ends were filled using a Klenow fragment of the *E. coli* DNA polymerase I. The resulting DNA was cleaved by XbaI, and the large fragment comprising the pPIC vector was isolated. Analogous thereto the DNA of pDISC3x19-LL was cleaved by NcoI and treated with a Klenow fragment. Following the cleavage using XbaI a small fragment, comprising a gene coding for the bivalent F_v antibody, was isolated. Its ligation with a pPIC-derived vector-DNA resulted in the plasmid pPIC-DISC-LL. The nucleotide and protein sequences of the bivalent F_v antibody construct are shown in FIGURE 8.

C. Example 3: Expression of the Tetravalent And/or Bivalent F_v Antibody Construct in Bacteria

E. coli XL1-blue cells (Strategene, La Jolla, CA) which had been transformed with the expression plasmids pDISC3x19-Ll and pDISC3x19-SL, respectively, were cultured overnight in 2xYT medium with 50 μg/ml ampicillin and 100 mM glucose (2xYT$_{Ga}$) at 37°C. 1:50 dilutions of the overnight cultures in 2xYT$_{GA}$ were cultured as flask cultures at 37°C while shaking with 200 rpm. When the cultures had reached an OD_{600} value of 0.8, the bacteria were pelleted by 10-minute centrifugation with 1500 g at 20°C and resuspended in the same volume of a fresh 2xYT medium containing 50 μg ampicillin and 0.4 M saccharose. IPTG was added up to a final concentration of 0.1 mM, and the growth was continued at room temperature (20-22°C) for 18 - 20 h. The cells were harvested by 10-minute centrifugation with 5000 g at 4°C. The culture supernatant was held back and stored on ice. In order to isolate the soluble periplasmic proteins, the pelleted bacteria were resuspended in 5 % of the initial volume of ice-cold 50 mM Tris_HCl, 20 % saccharose, 1 mM EDTA, pH 8.0. Following 1 hour of incubation on ice with occasional stirring the spheroplasts were centrifuged with 30,000 g at 4°C for 30 minutes, the soluble periplasmic extract being obtained as supernatant and the spheroplasts with the insoluble periplasmic material being obtained as pellet. The culture supernatant and the soluble periplasmic extract were combined and clarified by further centrifugation (30,000 g, 4°C, 40 min.). The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70 % saturation). The protein precipitate was obtained by centrifugation (10,000 g, 4°C, 40 min.) and dissolved in 10 % of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. An immobilized metal affinity chromatography (IMAC) was carried out at 4°C using a 5 ml column of chelating sepharose (Pharmacia) which was charged with Cu^{2+} and had been equilibrated with 50 mM Tris-HC1, 1 M NaCl, pH 7.0 (starting buffer). The sample was loaded by passing it over the column. It was then washed with twenty column volume of starting buffer, followed by starting buffer with 50 mM imidazole until the absorption at 280 nm of the effluent was at a minimum (about thirty column volumes). The absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0.

The protein concentrations were determined with the Bradford dye binding test (Bradford, 1976, *Anal. Biochem.* 72:248-254) using the Bio-Rad (Munich, Germany) protein assay kit. The concentrations of the purified tetravalent and bivalent F_v antibody constructs were determined from the A_{280} values using the extinction coefficients $\epsilon^{1mg/ml} = 1.96$ and 1.93, respectively.

D. Example 4: Expression of the Tetravalent And/or Bivalent Antibody Construct in the Yeast *Pichia Pastoris*

Competent *P. pastoris* GS155 cells (Invitrogen) were electroporated in the presence of 10 μg plasmid-DNA of pPIC-DISC-LL and pPIC-DISC-SL, respectively, which had been linearized with SacI. The transformants were selected for 3 days at 30°C on YPD plates containing 100 μg Zeocin™. The clones which secreted the bivalent and/or tetravalent F_v antibody constructs were selected by plate screening using an anti-c-myc-mAK 9E10 (IC chemikalien, Ismaning, Germany).

For the expression of the bivalent F_v antibody constructs and tetravalent F_v antibody constructs, respectively, the clones were cultured in YPD medium in shaking flasks for 2 days at 30°C with stirring. The cells were centrifuged resuspended in the same volume of the medium containing methanol and incubated for another 3 days at 30°C with stirring. The supernatants were obtained after the centrifguation. The recombinant product was isolated by ammonium sulfate precipitation, followed by IMAC as described above.

E. Examples 5: Characterization of the Tetravalent F_v Antibody Construct and Bivalent F_v Antibody Construct, Respectively

(A) Size exclusion chromatography

An analytical gel filtration of the F_v antibody constructs was carried out in PBS using a superdex 200-HR10/30 column (Pharmacia). The sample volume and the flow rate were 200 μl/min and 0.5 ml/min, respectively. The column was calibrated with high-molecular and low-molecular gel filtration calibration kits (Pharmacia).

(B) Flow cytometry

The human $CD3^+/CD19^-$ -acute T-cell leukemia line Jurkat and the $CD19^+/CD3^-$ B-cell line JOK-1 were used for flow cytometrie. 5×10^5 cells in 50 μl RPMI 1640 medium

(GIBCO BRL, Eggestein, Germany) which was supplemented with 10 % FCS and 0.1 % sodium azide (referred to as complete medium) were incubated with 100 μl of the F_v antibody preparations for 45 minute on ice. After washing using the complete medium the cells were incubated with 100 μl 10 μg/ml anti-c-myc-Mak 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second wash cycle, the cells were incubated with 100 μl of the FITC-labeled goat-anti-mouse-IgG (GIBCO BRL) under the same conditions as before. The cells were then washed again and resuspended in 100 μl 1 μg/ml propidium iodide solution (Sigma, Deisenhofen, Germany) in complete medium with the exclusion of dead cells. The relative fluorescence of the stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

(C) *Cytotoxicity text*

The CD19-expressing Burkitt lymphoma cell line Raji and Namalwa were used as target cells. The cells were incubated in RPMI (GIBCO BRL) which was supplemented with 10 % heat-inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate, at 37°C in a dampened atmosphere with 7.5 % CO_2. The cytotoxic T-cell tests were carried out in RPMI-1640 medium supplemented with 10 % FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. The cytotoxic activity was evaluated using a standard [^{51}Cr] release test; 2 x 10^6 target cells were labeled with 200 μCi Na[^{51}Cr]O_4 (Amersham-Buchler, Braunschweig, Germany) and washed 4 times and then resuspended in medium in a concentration of 5 x 10^6/ml. Increasing amounts of CTLs in 100 μl were titrated to 10^4 target cells/well or cavity in 50 μl. 50 μl antibodies were added to each well. The entire test was prepared three times and incubated at 37°C for 4 h. 100 μl of the supernatant were collected and tested for [^{51}Cr] release in a gamma counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). The maximum release was determined by incubation of the target cells in 10 % SDS, and the spontaneous release was determined by incubation of the cells in medium alone. The specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100.

F. Examples 6: Construction of the Plasmids Pdisc5-ll and Pdisc5-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Bacteria by High Cell Density Fermentation

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the skp/OmpH periplasmic factor for a greater production of recombinant antibodies. The skp gene was amplified by PCR using the primers skp-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G and skp-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G using the plasmid pGAH317 (Holck and Kleppe, 1988, *Gene* 67:117-124). The resulting PCR fragment was cleaved by Af1II and HindIII and inserted in the Af1II/HindIII-linearized plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers fe-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC and fe-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G. The XbaI/AflII-cleaved PCR fragments were inserted in pSKK before the skp insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the lac promoter/operator system (FIGURES 9 and 10).

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A multivalent F_v antibody construct having at least four variable domains which are linked with one another via the peptide linkers 1, 2 and 3.

2. The F_v antibody construct according to claim 1, wherein the peptide linkers 1 and 3 have 0 to 10 amino acids.

3. The F_v antibody construct according to claim 2, wherein the peptide linkers 1 and 3 have the amino acid sequence GG.

4. The F_v antibody construct according to any of claims 1 to 3, wherein the F_v antibody construct is bivalent.

5. The F_v antibody construct according to claim 4, wherein the peptide linker 2 has 11 to 20 amino acids.

6. The F_v antibody construct according to claim 4 or 5, wherein the peptide linker 2 has the amino acid sequence $(G_4S)_4$

7. The F_v antibody construct according to any of claims 1 to 3, wherein the F_v antibody construct is tetravalent.

8. The F_v antibody construct according to claim 7, wherein the peptide linker 2 has 3 to 10 amino acids

9. The F_v antibody construct according to claim 7 or 8, wherein the peptide linker 2 comprises the amino acid sequence GGPGS.

10. The F_v antibody construct according to any of claims 1 to 9, wherein the F_v antibody construct is multispecific.

11. F_v antibody construct according to claim 10, wherein the F_v antibody construct is bispecific.

12. The F_v antibody construct according to any of claims 1 to 9, wherein the F_v antibody construct is monospecific.

13. A method of producing the multivalent F_v antibody construct according to any of claims 1 to 12, wherein DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid.

14. Expression plasmid coding for the multivalent F_v antibody construct according to any of claims 1 to 12.

15. The expression plasmid according to claim 14, namely pDISC3x19-LL.

16. The expression plasmid according to claim 14, namely pDISC3x19-SL.

17. The expression plasmid according to claim 14, namely pPIC-DISC-LL.

18. The expression plasmid according to claim 14, namely pPIC-DISC-SL.

19. The expression plasmid according to claim 14, namely pDISC5-LL.

20. The expression plasmid according to claim 14, namely pDISC5-SL.

21. Use of the multivalent F_v antibody construct according to any of claims 1 to 12 for the diagnosis and/or treatment of diseases.

22. Use according to claim 21, wherein he diseases are viral, bacterial or tumoral diseases.

Claims As Amended In Response To Written Opinion

1. A multivalent F_V antibody construct having at least four variable domains which are linked with one another via the peptide linkers 1, 2 and 3, wherein the peptide linkers 1 and 3 have 0 to 10 amino acids.

2. The F_V antibody construct according to claim 1, wherein the peptide linkers 1 and 3 have the amino acid sequence GG.

3. The F_V antibody construct according to claim 1 or 2, wherein the F_V antibody construct is bivalent.

4. The F_V antibody construct according to claim 3, wherein the peptide linker 2 has 11 to 20 amino acids.

5. The F_V antibody construct according to claim 3 or 4, wherein the peptide linker 2 has the amino acid sequence $(G_4S)_4$.

6. The F_V antibody construct according to claim 1 or 2, wherein the F_V antibody construct is tetravalent.

7. The F_V antibody construct according to claim 6, wherein the peptide linker 2 has 3 to 10 amino acids.

8. The F_V antibody construct according to claim 6 or 7, wherein the peptide linker 2 comprises the amino acid sequence GGPGS.

9. The F_V antibody construct according to any of claims 1 to 8, wherein the F_V antibody construct is multispecific.

10. F_V antibody construct according to claim 9, wherein the F_V antibody construct is bispecific.

11. The F_V antibody construct according to any of claims 1 to 8, wherein the F_V antibody construct is monospecific.

12. A method of producing the multivalent F_V antibody construct according to any of claims 1 to 11, wherein DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_V antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid.

13. Expression plasmid coding for the multivalent F_V antibody construct according to any of claims 1 to 11.

14. The expression plasmid according to claim 13, namely pDISC3x19-LL.

15. The expression plasmid according to claim 13, namely pDISC3x19-SL.

16. The expression plasmid according to claim 13, namely pPIC-DISC-LL.

17. The expression plasmid according to claim 13, namely pPIC-DISC-SL.

18. The expression plasmid according to claim 13, namely pDISC5-LL.

19. The expression plasmid according to claim 13, namely pDISC6-SL.

20. Use of the multivalent F_V antibody construct according to any of claims 1 to 11 for the diagnosis and/or treatment of diseases.

21. Use according to claim 20, wherein the diseases are viral, bacterial or tumoral diseases.

ABSTRACT

The present invention relates to a multivalent F_v antibody construct having at least four variable domains which are linked with each over via the peptide linkers 1, 2 and 3. The invention also concerns expression plasmids which code for such an F_v antibody construct and a method of producing the F_v antibody constructs as well as their use.

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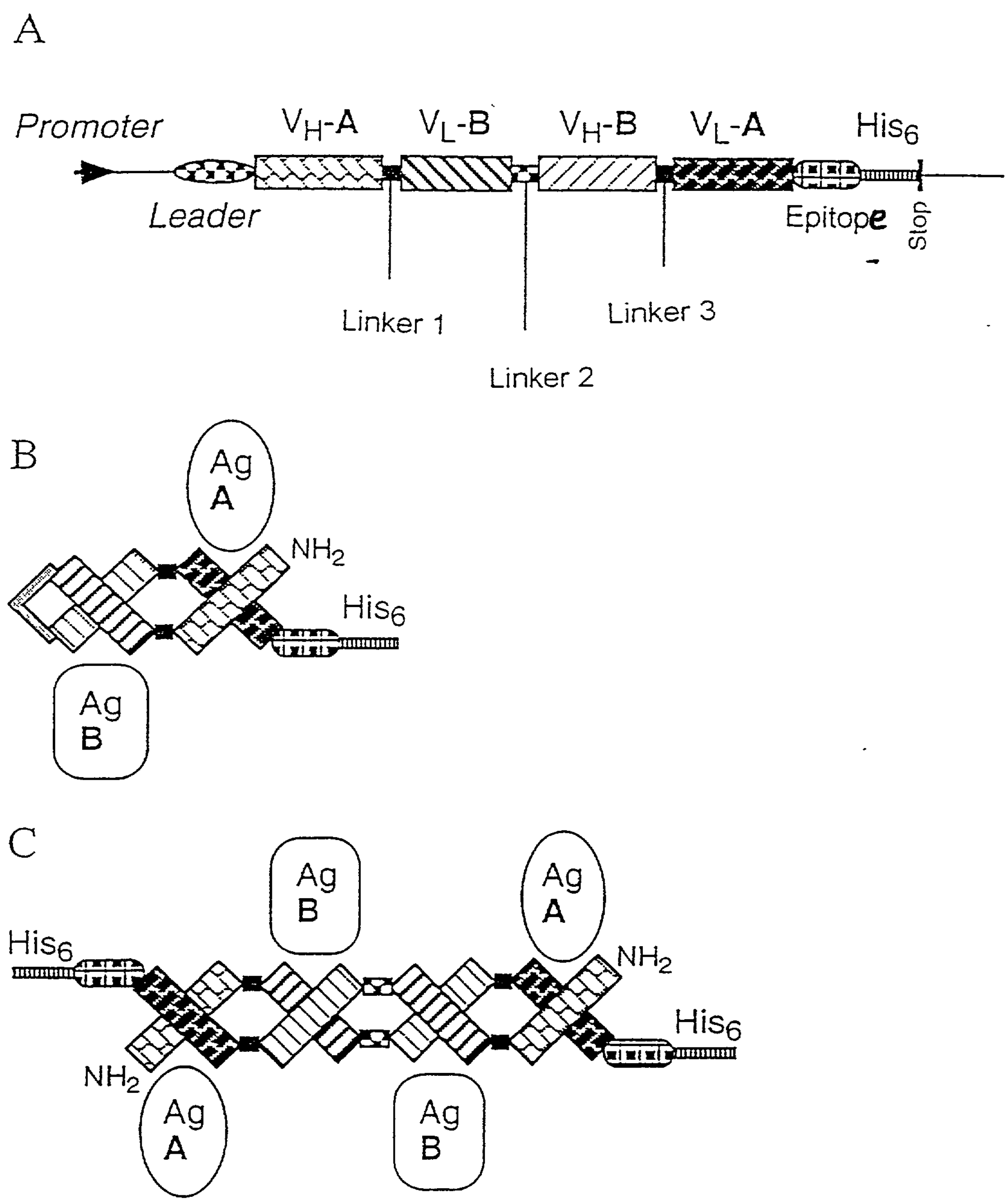
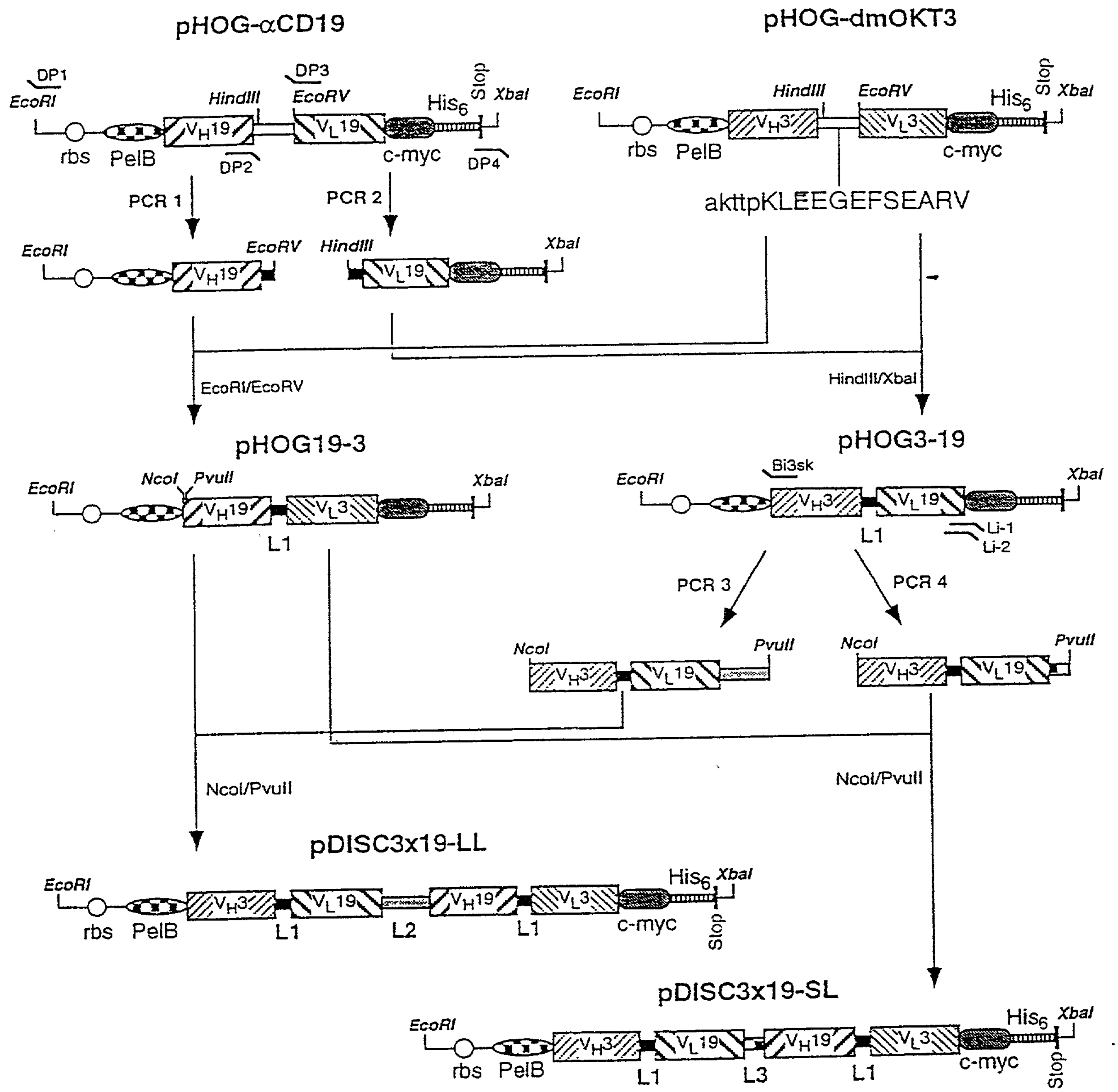


FIGURE 1



Linkers: L1 = GG
L2 = $(G_4S)_4$
L3 = GGPGS

FIGURE 2

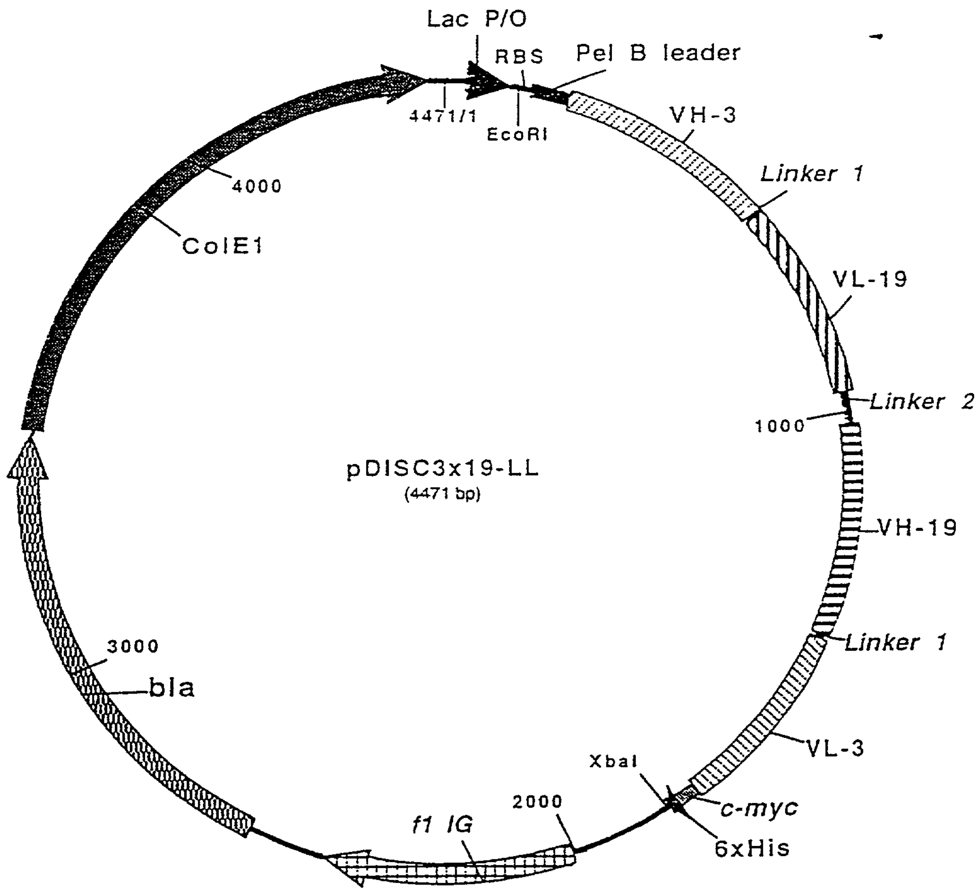

Lac P/O
RBS
Pel B leader
4471/1
EcoRI
VH-3
Linker 1
4000
ColE1
VL-19
Linker 2
1000
pDISC3x19-LL
(4471 bp)
VH-19
Linker 1
3000
bla
VL-3
XbaI
c-myc
6xHis
2000
f1 IG

FIGURE 3

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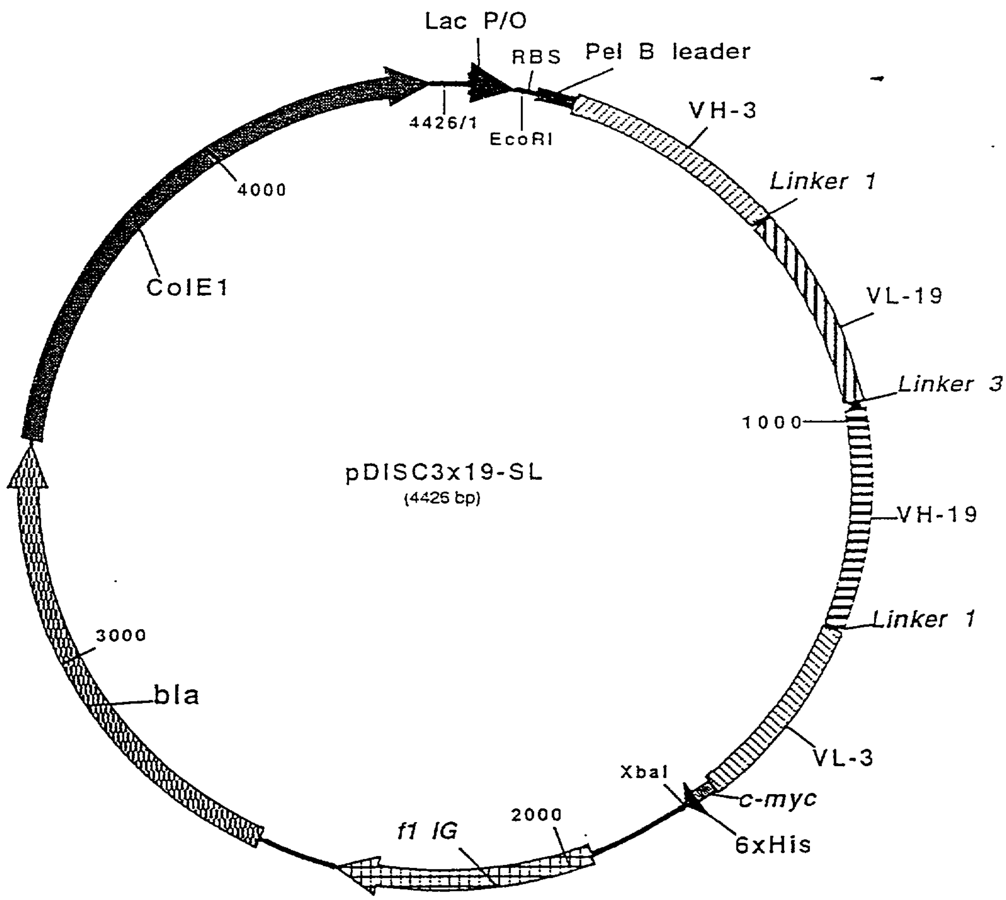

FIGURE 4

EcoRI RBS PelB leader NcoI
1 GAATTCATTAAAGAGGAGAAATTAACCATGAAATACCTATTGCCTACGGCAGCCGCTGGCTTGCTGCTGCTGGCAGCTCAGCCGGCCATGG
1▸ M K Y L L P T A A A G L L L L A A Q P A M

Frame-H1 VH anti-CD3
92 CGCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTAC
22▸ A Q V Q L Q Q S G A E L A R P G A S V K M S C K A S G Y T F T

CDR-H1 Frame-H2 CDR-H2
183 TAGGTACACGATGCACTGGGTAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATAC
52▸ R Y T M H W V K Q R P G Q G L E W I G Y I N P S R G Y T

Frame-H3
257 TAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGACTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGAC
30▸ N Y N Q K F K D K A T L T T D K S S S T A Y M Q L S S L T

CDR-H3 Frame-H4
354 ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGATATTATGATGATCATTACAGCCTTGACTACTGGGGCCAAGGCACCACTCTCA
109▸ S E D S A V Y Y C A R Y Y D D H Y S L D Y W G Q G T T L

CH1 *Linker 1* Frame-L1 VL anti-CD19
440 CAGTCTCCTCAGCCAAAACAACACCCAAGCTT*GGCGG*TGATATCTTGCTCACCCAAACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGA
138▸ T V S S A K T T P K L G G D I L L T Q T P A S L A V S L G Q

CDR-L1 Frame-L2
530 GGGCCACCATCTCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACAGATTCCAGGAC
168▸ R A T I S C K A S Q S V D Y D G D S Y L N W Y Q Q I P G

CDR-L2 Frame-L3
614 AGCCACCCAAACTCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTT
196▸ Q P P K L L I Y D A S N L V S G I P P R F S G S G S G T D F

CDR-L3 Frame-L4
702 CACCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCTATCACTGTCAGCAAAGTACTGAGGATCCGTGGACGTTCGGTGGA
225▸ T L N I H P V E K V D A A T Y H C Q Q S T E D P W T F G G

C kappa NotI *Linker 2*
790 GGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCGGCCGCT*GGTGGTGGTGGTTCTGGCGGCGGTGGTAGCGGTGGTGGCGGC*
255▸ G T K L E I K R A D A A A A G G G G S G G G G S G G G G

PvuII Frame-H1 VH anti-CD19
874 *TCCGGTGGTGGTGGTAGC*CAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCCTCAGTGAAGATTTCCTGCAAGG
283▸ S G G G G S Q V Q L Q Q S G A E L V R P G S S V K I S C K

CDR-H1 Frame-H2 CDR-H2
962 CTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGCCTGGACAGGGTCTTGAGTGGATTGGACAGATTTGGC
312▸ A S G Y A F S S Y W M N W V K Q R P G Q G L E W I G Q I W

PstI Frame-H3
1049 CTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGTAAAGCCACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACA
341▸ P G D G D T N Y N G K F K G K A T L T A D E S S S T A Y

CDR-H3
1133 TGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGACGGGAGACTACGACGGTAGGCCGTTATTACTAT
369▸ M Q L S S L A S E D S A V Y F C A R R E T T T V G R Y Y Y

Frame-H4 CH1 *Linker 1* Frame-L1
1219 GCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACAACACCCAAGCTT*GGCGG*TGATATCGTGCTCACTC
398▸ A M D Y W G Q G T S V T V S S A K T T P K L G G D I V L T .

VL anti-CD3 CDR-L1
1307 AGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGG
427▸ Q S P A I M S A S P G E K V T M T C S A S S S V S Y M N W

Frame-L2 CDR-L2 Frame-L3
1393 TACCAGCAGAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCACTTCAGGGGCA
456▸ Y Q Q K S G T S P K R W I Y D T S K L A S G V P A H F R G

CDR-L3
1481 GTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAA
485▸ S G S G T S Y S L T I S G M E A E D A A T Y Y C Q Q W S S N

Frame-L4 C kappa *c-myc epitope*
1569 CCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGCACCAACTGGATCC*GAACAAAAGCTGATCTCAG*
514▸ P F T F G S G T K L E I N R A D T A P T G S E Q K L I S

His6 tail XbaI
1655 *AAGAAGACCTAAACTCA*CATCACCATCACCATCACTAATCTAGA
543▸ E E D L N S H H H H H H •

FIGURE 5

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EcoRI        RBS          PelB leader                                                   NcoI
   1 GAATTCATTAAAGAGGAGAAATTAACCATGAAATACCTATTGCCTACGGCAGCCGCTGGCTTGCTGCTGCTGGCAGCTCAGCCGGCCATGG
                                1▸ M  K  Y  L  L  P  T  A  A  A  G  L  L  L  L  A  A  Q  P  A  M
                          Frame-H1                                        VH  anti-CD3
  92 CGCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTAC
 22▸ A  Q  V  Q  L  Q  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T
       CDR-H1          Frame-H2                                        CDR-H2
 183 TAGGTACACGATGCACTGGGTAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATAC
 52▸  R  Y  T  M  H  W  V  K  Q  R  P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  R  G  Y  T
                                   Frame-H3
 267 TAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGACTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGAC
 80▸  N  Y  N  Q  K  F  K  D  K  A  T  L  T  T  D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T
                                              CDR-H3                        Frame-H4
 354 ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGATATTATGATGATCATTACAGCCTTGACTACTGGGGCCAAGGCACCACTCTCA
109▸  S  E  D  S  A  V  Y  Y  C  A  R  Y  Y  D  D  H  Y  S  L  D  Y  W  G  Q  G  T  T  L
              CH1                 Linker 1     Frame-L1                          VL  anti-CD19
 440 CAGTCTCCTCAGCCAAAACAACACCCAAGCTTGGCGGTGATATCTTGCTCACCCAAACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGA
138▸ T  V  S  S  A  K  T  T  P  K  L  G  G  D  I  L  L  T  Q  T  P  A  S  L  A  V  S  L  G  Q
                       CDR-L1                                                     Frame-L2
 530 GGGCCACCATCTCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACAGATTCCAGGAC
168▸ R  A  T  I  S  C  K  A  S  Q  S  V  D  Y  D  G  D  S  Y  L  N  W  Y  Q  Q  I  P  G
                          CDR-L2                                Frame-L3
 614 AGCCACCCAAACTCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTT
196▸ Q  P  P  K  L  L  I  Y  D  A  S  N  L  V  S  G  I  P  P  R  F  S  G  S  G  S  G  T  D  F
                                                                 CDR-L3              Frame-L4
 702 CACCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCTATCACTGTCAGCAAAGTACTGAGGATCCGTGGACGTTCGGTGGA
225▸  T  L  N  I  H  P  V  E  K  V  D  A  A  T  Y  H  C  Q  Q  S  T  E  D  P  W  T  F  G  G
                       C kappa     NotI          Linker 3                    PvuII     Frame-H1
 790 GGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCGGCCGCTGGTGGCCCAGGGTCGCAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCT
255▸ G  T  K  L  E  I  K  R  A  D  A  A  A  A  G  G  P  G  S  Q  V  Q  L  Q  Q  S  G  A  E  L
   VH  anti-CD19                                             CDR-H1                Frame-H2
 879 GGTGAGGCCTGGGTCCTCAGTGAAGATTTCCTGCAAGGCTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGC
284▸  V  R  P  G  S  S  V  K  I  S  C  K  A  S  G  Y  A  F  S  S  Y  W  M  N  W  V  K  Q  R
                                    CDR-H2
 968 CTGGACAGGGTCTTGAGTGGATTGGACAGATTTGGCCTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGTAAAGCC
314▸ P  G  Q  G  L  E  W  I  G  Q  I  W  P  G  D  G  D  T  N  Y  N  G  K  F  K  G  K  A
                 Frame-H3
1051 ACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGAC
342▸ T  L  T  A  D  E  S  S  S  T  A  Y  M  Q  L  S  S  L  A  S  E  D  S  A  V  Y  F  C  A  R
          CDR-H3                                          Frame-H4                          CH1
1142 GGGAGACTACGACGGTAGGCCGTTATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAA
372▸ R  E  T  T  T  V  G  R  Y  Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S  A  K
                Linker 1      Frame-L1       VL  anti-CD3
1226 CAACACCCAAGCTTGGCGGTGATATCGTGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCA
400▸ T  T  P  K  L  G  G  D  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  M  T  C
          CDR-L1                            Frame-L2                                      CDR-L2
1316 GTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCAGAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGACACATCCAA
430▸ S  A  S  S  S  V  S  Y  M  N  W  Y  Q  Q  K  S  G  T  S  P  K  R  W  I  Y  D  T  S  K
                  Frame-L3
1401 ACTGGCTTCTGGAGTCCCTGCTCACTTCAGGGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGC
458▸  L  A  S  G  V  P  A  H  F  R  G  S  G  S  G  T  S  Y  S  L  T  I  S  G  M  E  A  E  D  A
                   CDR-L3                            Frame-L4                        C kappa
1491 TGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGC
488▸  A  T  Y  Y  C  Q  Q  W  S  S  N  P  F  T  F  G  S  G  T  K  L  E  I  N  R  A  D  T  A
                 c-myc  epitope                                    His6 tail        XbaI
1578 ACCAACTGGATCCGAACAAAAGCTGATCTCAGAAGAAGACCTAAACTCACATCACCATCACCATCACTAATCTAGA
517▸  P  T  G  S  E  Q  K  L  I  S  E  E  D  L  N  S  H  H  H  H  H  H  •
```

FIGURE 6

```
941  ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTAC
  1▸ M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T

                                         alpha-factor signal
1015 AACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATG
 25▸ T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D

1089 TTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
 50▸ V  A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A

                                                    EcoRI
                          XhoI           ◆     ◆
1163 GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCCAGGTGCAACTGCAGCAGTC
 75▸ A  K  E  E  G  V  S  L  E  K  R  E  A  E  A  E  F  Q  V  Q  L  Q  Q  S

   VH anti-CD3
1234 TGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
 98▸ G  A  E  L  A  R  P  G  A  S  V  K  M  S  C  K  A  S
```

FIGURE 7

941 ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTAC
1▸ M R F P S I F T A V L F A A S S A L A A P V N T T

alpha-factor signal

1015 AACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATG
25▸ T E D E T A Q I P A E A V I G Y S D L E G D F D

BsrDI

1089 TTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
50▸ V A V L P F S N S T N N G L L F I N T T I A S I A

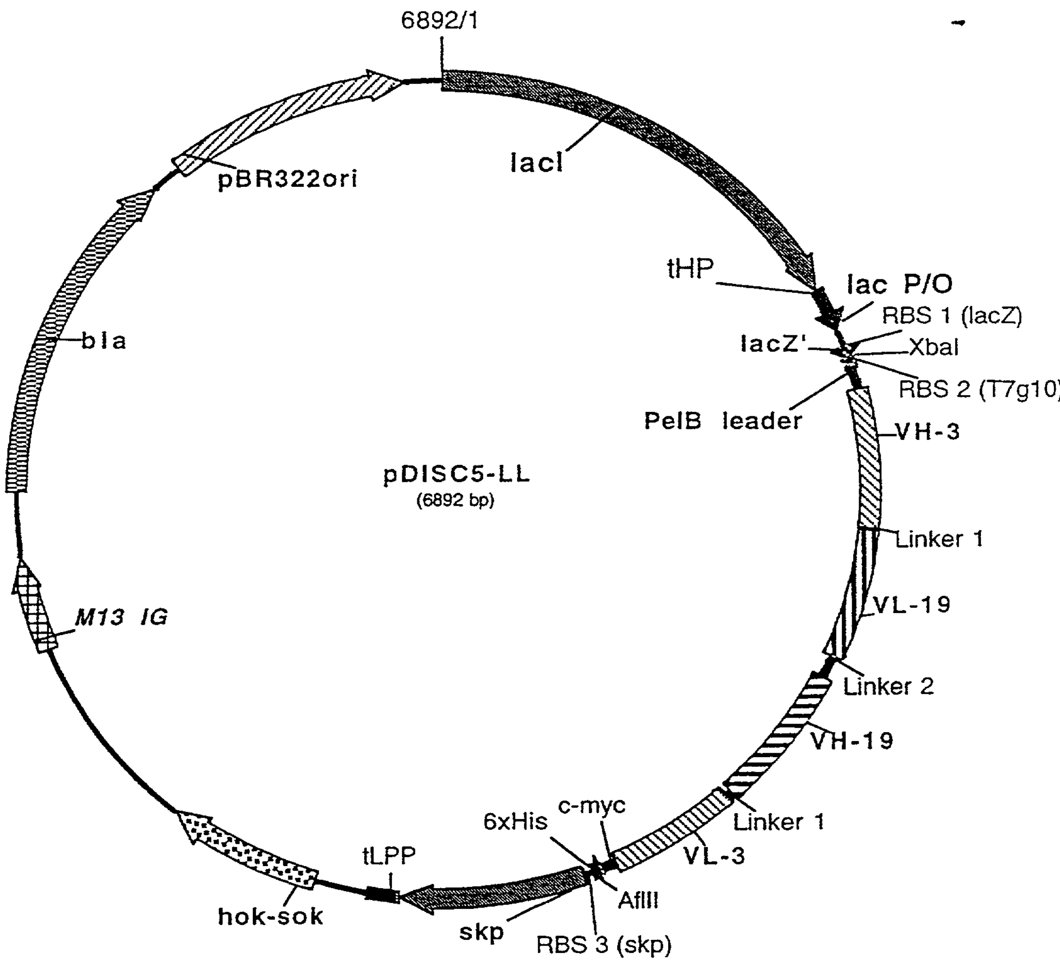
XhoI EcoRI

1163 GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCATGGCGCAGGTGCAACTGCAG
75▸ A K E E G V S L E K R E A E A E F M A Q V Q L Q

VH anti-CD3

1235 CAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
99▸ Q S G A E L A R P G A S V K M S C K A S

FIGURE 8



6892/1
lacI
pBR322ori
tHP
lac P/O
RBS 1 (lacZ)
lacZ'
XbaI
RBS 2 (T7g10)
PelB leader
VH-3
bla
pDISC5-LL
(6892 bp)
Linker 1
VL-19
M13 IG
Linker 2
VH-19
c-myc
6xHis
Linker 1
VL-3
tLPP
AflII
hok-sok
skp
RBS 3 (skp)

FIGURE 9

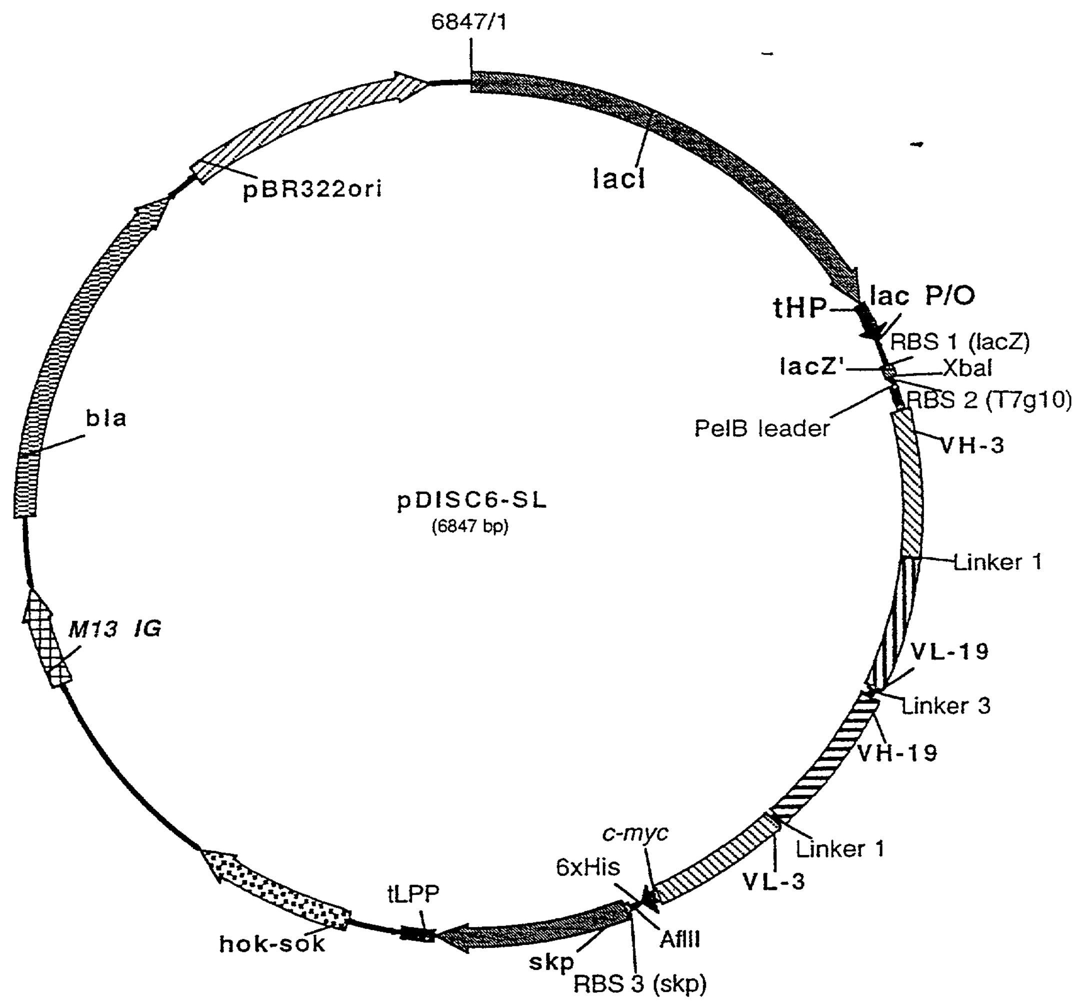

6847/1
lacI
pBR322ori
tHP
lac P/O
RBS 1 (lacZ)
lacZ'
XbaI
RBS 2 (T7g10)
PelB leader
VH-3
bla
pDISC6-SL
(6847 bp)
Linker 1
M13 IG
VL-19
Linker 3
VH-19
c-myc
6xHis
Linker 1
VL-3
tLPP
AflII
hok-sok
skp
RBS 3 (skp)

FIGURE 10

09/ 674794

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MULTIVALENT ANTIBODY CONSTRUCTS

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[Applicant: Deutsches Krebsforschungszentrum]

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MULTIVALENT ANTIBODY CONSTRUCTS

This is a national phase filing of the Application No. PCT/DE99/01350, which was filed with the Patent Corporation Treaty on May 5, 1999, and is entitled to priority of the German Patent Application 198 19 846.9, filed May 5, 1998.

I. FIELD OF THE INVENTION

The present invention relates to multivalent F_v antibody constructs, expression plasmids which code for them, and a method for producing the F_v antibody constructs as well as the use thereof.

II. BACKGROUND OF THE INVENTION

Natural antibodies are dimers and are therefore referred to as bivalent. They have four variable domains, namely two V_H domains and two V_L domains. The variable domains serve as binding sites for an antigen, a binding site being formed from a V_H domain and a V_L domain. Natural antibodies recognize one antigen each, so that they are also referred to as monospecific. Furthermore, they also have constant domains which add to the stability of the natural antibodies. On the other hand, they are also co-responsible for undesired immune responses which result when natural antibodies of various animal species are administered mutually.

In order to avoid such immune responses, antibodies are constructed which lack the constant domains. In particular, these are antibodies which only comprise the variable domains. Such antibodies are designated F_v antibody constructs. They are often available in the form of single-chain monomers paired with one another.

However, it showed that F_v antibody constructs only have little stability. Therefore, their usability for therapeutic purposes is strongly limited.

Thus, it is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

According to the invention this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a multivalent F_v antibody construct having at least four variable domains which are linked with each over via the peptide linkers 1, 2 and 3. The invention also concerns expression plasmids which code for such an F_v antibody construct and a method of producing the F_v antibody constructs as well as their use.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the genetic organization of an F_v antibody construct (A) according to the invention and schemes for forming a bivalent (B) or tetravalent F_v antibody construct (C). Ag: antigen; His_6: six C-terminal histidine residues; stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

FIGURE 2 shows the scheme for the construction of the plasmids pDISC3x19-LL and pDISC3x19-SL. c-myc: sequence coding for an epitope which is recognized by the antibody 9E1, His_6: sequence which codes for six C-terminal histidine residues; Pe1B: signal peptide sequence of the bacterial pectate lyase (Pe1B leader); rbs: ribosome binding site; Stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

FIGURE 3 shows a diagram of the expression plasmid pDISC3x19-LL. 6xHis: sequence which cods for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; ColE1: origin of the DNA replication; f1; Lac P/Of: wt lac-operon promoter/operator; linker 1: sequence which codes for a GlyGly dipeptide linking the V_H and V_L domains; linker 2: sequence coding for a $(Gly_4Ser)_4$ polypeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 4 shows a diagram of the expression plasmid pDISC3x19-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for ß-lactamase which is responsible for the ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope recognized by the 9E10 antibody; ColE1: origin of DNA replication; fl-IG: intergenic region of the bacteriophage fl; Lac P/Of: wt lacoperon promoter/operator: linker 1: sequence which codes for a GyGly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 5 shows the nucleotide sequence and the amino acid sequence derived therefrom of the bivalent F_v antibody construct encoded by the expression plasmid pDIS3x19-LL. c-myc epitope: sequence coding for an epitope which is recognized by the antibody 9E10; CDR: region determining the complementarity; framework: framework region; His6 tail: sequence which codes for six C-terminal histidine residues; PelB leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 6 shows the nucleotide sequence and the derived amino acid sequence of the tetravalent F_v antibody construct encoded by the expression plasmid pDISC3x19-SL. c-myc epitope: sequence coding for an epitope which is recognized by the 9E10 antibody; CDR: region determining complementarity; framework: framework region; His6 tail: sequence coding for the six C-terminal histidine residues; PelB leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

FIGURE 7 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene which codes for an α-factor leader sequence and a gene coding for the tetravalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-SL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae-α* factor secretion signal; V_H: variable region of the heavy chain. Rhombs indicate the signal cleaving sites.

FIGURE 8 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene coding for an α-factor leader sequence and a gene which codes for the bivalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-LL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae-α* factor secretion signal; V_H: variable region of the heavy chain. Rhombs show the signal cleaving sites.

FIGURE 9 shows a diagram of the expression plasmid pDISC5-LL. 6xHis: sequence coding for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilizing DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon-promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which codes for a GlyGly dipeptide connecting the V_H and V_L domains; linker 2: sequence which codes for a $(Gly_4Ser)_4$ polypeptide linking the hybrid scFv fragments; M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader; signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site which originates from the *E. coli* LacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; V_H and V_L: variable region of the heavy and light chains.

FIGURE 10 shows a diagram of the expression plasmid pDISC6-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs: c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilized DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which cods for a Glygly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide linking the hybrid scFv fragments: M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site originating from the *E. coli* lacZ gene (lacZ), from the bacteriophage

T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; V_H and V_L: variable region of the heavy and light chains.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

Therefore, the subject matter of the present invention relates to a multivalent F_v antibody construct which has great stability. Such a construct is suitable for diagnostic and therapeutic purposes.

The present invention is based on the applicant's insights that the stability of an F_v antibody construct can be increased if it is present in the form of a single-chain dimer where the four variable domains are linked with one another via three peptide linkers. The applicant also recognized that the F_v antibody construct folds with itself when the middle peptide linker has a length of about 10 to 30 amino acids. The applicant also recognized that the F_v antibody construct folds with other F_v antibody constructs when the middle peptide linker has a length of about up to 10 amino acids so as to obtain a multimeric, *i.e.*, multivalent, F_v antibody construct. The applicant also realized that the F_v antibody construct can be multispecific.

According to the invention the applicant's insights are utilized to provide a multivalent F_v antibody construct which comprises at least four variable domains which are linked with one another via peptide linkers 1, 2 and 3.

The expression "F_v antibody construct" refers to an antibody which has variable domains but no constant domains.

The expression "multivalent F_v antibody construct" refers to an F_V antibody which has several, but at least four, variable domains. This is achieved when the single-chain F_v antibody construct folds with itself so as to give four variable domains, or folds with other single-chain F_v antibody constructs. In the latter case, an F_v antibody construct is given which has 8, 12, 16, etc., variable domains. It is favorable for the F_v antibody construct to have four or eight variable domains, *i.e.*, it is bivalent or tetravalent [(cf. Fig.](FIGURE 1).

Furthermore, the variable domains may be equal or differ from one another, so that he antibody construct recognizes one or several antigens. The antibody construct preferably recognizes one or two antigens, *i.e.*, it is monospecific and bispecific, respectively. Examples of such antigens are proteins CD19 and CD3.

The expression "peptide linkers 1, 3" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linkers 1 and 3 may be equal or differ from each other. Furthermore, the peptide linker may have a length of about 0 to 10 amino acids. In the former case, the peptide linker is only a peptide bond from the COOH residue of one of the variable domains and the NH_2 residue of another of the variable domains. The peptide linker preferably comprises the amino acid sequence GG.

The expression "peptide linker 2" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linker may also have a length of about 3 to 10 amino acids, in particular 5 amino acids, and most particularly the amino acid sequence GGPGS, which serves for achieving that the single-chain F_v antibody constructs. The peptide linker can also have a length of about 11 to 20 amino acids, in particular 15 to 20 amino acids, and most particularly the amino acid sequence $(G_4S)_4$, which serves for achieving that the single-chain F_v antibody construct folds with itself.

An F_v antibody constructs according to the invention can be produced by common methods. A method is favorable in which DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid. Reference is made to Example 1 to 6. As to the expressions "F_v antibody construct" and "peptide linker" reference is made to the above explanations and, by way of supplement, to Maniatis, T. *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

DNAs which code for an F_v antibody construct according to the invention also represent a subject matter of the present invention. Furthermore, expression plasmids which

contain such DNAs also represent a subject matter of the present invention. Preferred expression plasmids are pDISC3x19-LL, pDISC3x19-SL, pPIC-DISC-LL, pPIC-DISC-SL, pDISC5-LL and pDISC6-SL. The first four were deposited with the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellen) [German-type collection for micro-organisms and cells] on April 30, 1998 under DSM 12150, DSM 12149, DSM 12152 and DSM 12151, respectively.

Another subject matter of the present invention relates to a kit, comprising:

(a) an F_v antibody construct according to the invention, and/or

(b) an expression plasmid according to the invention, and

(c) conventional auxiliary agents, such as buffers, solvents and controls.

One or several representatives of the individual components may be present.

The present invention provides a multivalent F_v antibody construct where the variable domains are linked with one another via peptide linkers. Such an antibody construct distinguishes itself in that it contains no parts which can lead to undesired immune reactions. Furthermore, it has great stability. It also enables to bind several antigens simultaneously. Therefore, the F_v antibody construct according to the invention is perfectly adapted to be used not only for diagnostic but also for therapeutic purposes. Such purposes can be seen as regards any disease, in particular a viral, bacterial or tumoral disease.

[Brief description of the drawings:

Fig. 1 shows the genetic organization of an Fv antibody construct (A) according to the invention and schemes for forming a bivalent (B) or tetravalent Fv antibody construct (C). Ag: antigen; His6: six C-terminal histidine residues; stop: stop codon (TAA); VH and VL: variable region of the heavy and light chains.

Fig 2 shows the scheme for the construction of the plasmids pDISC3x19-LL and pDISC3x19-SL. c-myc: sequence coding for an epitope which is recognized by the antibody 9E1, His6: sequence which codes for six C-terminal histidine residues; Pe1B: signal peptide sequence of the bacterial pectate lyase (Pe1B leader); rbs: ribosome binding site; Stop: stop codon (TAA); VH and VL: variable region of the heavy and light chains.

Fig. 3 shows a diagram of the expression plasmid pDISC3x19-LL. 6xHis: sequence which cods for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; ColE1: origin of the DNA replication; f1; Lac P/Of: wt lac-operon promoter/operator; linker 1: sequence which codes for a GlyGly dipeptide linking the VH and VL domains; linker 2: sequence coding for a (Gly4Ser)4 polypeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; VH and VL: variable region of the heavy and light chains.

Fig. 4 shows a diagram of the expression plasmid pDISC3x19-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for ß-lactamase which is responsible for the ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope recognized by the 9E10 antibody; ColE1: origin of DNA replication; f1-IG: intergenic region of the bacteriophage f1; Lac P/Of: wt lacoperon promoter/operator: linker 1: sequence which codes for a GyGly dipeptide which links the VH and VL domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide which links the hybrid scFv fragments; Pe1-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; VH and VL: variable region of the heavy and light chains.

Fig. 5 shows the nucleotide sequence and the amino acid sequence derived therefrom of the bivalent Fv antibody construct encoded by the expression plasmid pDIS3x19-LL. c-myc epitope: sequence coding for an epitope which is recognized by the antibody 9E10; CDR: region determining the complementarity; framework: framework region; His6 tail: sequence which codes for six C-terminal histidine residues; Pe1B leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; VH and VL: variable region of the heavy and light chains.

Fig 6 shows the nucleotide sequence and the derived amino acid sequence of the tetravalent Fv antibody construct encoded by the expression plasmid pDISC3x19-SL. c-myc epitope: sequence coding for an epitope which is recognized by the 9E10 antibody; CDR: region determining complementarity; framework: framework region; His6 tail: sequence coding for

the six C-terminal histidine residues; Pe1B leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; VH and VL: variable region of the heavy and light chains.

Fig. 7 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene which codes for an α-factor leader sequence and a gene coding for the tetravalent Fv antibody construct in the Pichia expression plasmid pPIC-DISC-SL. Alpha-factor signal: leader peptide sequence of the Saccharomyces cerevisiae-α factor secretion signal; VH: variable region of the heavy chain. Rhombs indicate the signal cleaving sites.

Fig. 8 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene coding for an α-factor leader sequence and a gene which codes for the bivalent Fv antibody construct in the Pichia expression plasmid pPIC-DISC-LL. Alpha-factor signal: leader peptide sequence of the Saccharomyces cerevisiae-α factor secretion signal; VH: variable region of the heavy chain. Rhombs show the signal cleaving sites.

Fig. 9 shows a diagram of the expression plasmid pDISC5-LL. 6xHis: sequence coding for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilizing DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon-promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which codes for a GlyGly dipeptide connecting the VH and VL domains; linker 2: sequence which codes for a (Gly4Ser)4 polypeptide linking the hybrid scFv fragments; M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pe1-B leader; signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site which originates from the E. coli LacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the E. coli skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; VH and VL: variable region of the heavy and light chains.

Fig. 10 shows a diagram of the expression plasmid pDISC6-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for β-lactamase responsible for ampicillin resistance; bp: base pairs: c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilized DNA locus; LacI: gene which codes for the Lac repressor; Lac P/Of: wt lac-operon promoter/operator; LacZ': gene which codes for the α-peptide of β-galactosidase; linker 1: sequence which cods for a Glygly dipeptide which links the VH and VL domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide linking the hybrid scFv fragments: M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pe1-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site originating from the E. coli lacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the E. coli skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; VH and VL: variable region of the heavy and light chains.

The invention is explained by the below examples.

Example 1: Construction of the plasmids pDISC3x19-LL and pDISC3x9-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific Fv antibody constructs in bacteria.]

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Construction of the Plasmids Pdisc3x19-ll and Pdisc3x9-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Bacteria

The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov *et al.*, 1996, *J.-Immunol. Meth.* 196[,]:51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov *et al.*, 1997, *Protein Eng.* 10[,]:445-453), respectively, were used for the construction of expression plasmids for a single-chain F_v antibody construct. A PCR fragment 1 of the V_H domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-TTAGATCTATTAAAGAGGAGAAATTAACC, and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC [(cf. Fig](FIGURE. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR fragment 2 of the V_L domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidinyl tail, was produced using the primers DP3, 5;-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA, and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-GGTGATGTGAGT TTAGG. The PCR fragment 2 was cleaved by HindIII and XbaI and ligated with the HindIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 [(cf. Fig.](FIGURE 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG and either Li-1, 5'-TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-CCACCACCG CTACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATCAGCCCG, for the production of a long flexible $(Gly_4Ser)_4$ inter-scFV linker (PCR fragment 3, [cf. Fig.] FIGURE 2) or Li-2, 5'-TATATA-CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATCA GCCG, for the production of a short rigid GGPGS linker (PCR fragment 4, [cf. Fig.] FIGURE 2). The expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the NcoI/PvuII restriction fragment from pHOG19-3, comprising the

vector framework and the NcoI/PVuII-cleaved PCR fragments 3 and 4, respectively [(cf. Figs.](FIGURES 3[,] and 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F_v antibody constructs are indicated in [Figs] FIGURES 5 and 6, respectively.

B. Example 2: Construction of the [plasmids pPIC-DISC-LL and pPIC-DISC-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific Fv antibody constructs in yeast] Plasmids Ppic-disc-ll and Ppic-disc-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Yeast

(A) Construction of pPIC-DISC-SL

The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal, followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli*. The gene which codes for the tetravalent F_v antibody construct (scDia-SL) was amplified by means of PCR by the template pDIC3x19-SL using the primers 5-PIC, 5'-CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG. The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences of the tetravalent F_v antibody construct are shown in [Fig.] FIGURE 7.

(B) Construction of pPIC-DISC-LL

The construction of pPIC-DISC-LL was carried out on the basis of pPICZαA (Invitrogen BV, Leek, Netherlands) and pDISC3x19-LL [(cf. Fig.](FIGURE 3). The plasmid -DNA pPICZαA was cleaved by EcoRI. The overhanging 5'-ends were filled using a Klenow fragment of the *E. coli* DNA polymerase I. The resulting DNA was cleaved by XbaI, and the large fragment comprising the pPIC vector was isolated. Analogous thereto the DNA of pDISC3x19-LL was cleaved by NcoI and treated with a Klenow fragment. Following the cleavage using XbaI a small fragment, comprising a gene coding for the

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bivalent F_v antibody, was isolated. Its ligation with a pPIC-derived vector-DNA resulted in the plasmid pPIC-DISC-LL. The nucleotide and protein sequences of the bivalent F_v antibody construct are shown in [Fig.] FIGURE 8.

C. Example 3: Expression of the [tetravalent and/or bivalent Fv antibody construct in bacteria] Tetravalent And/or Bivalent F_v Antibody Construct in Bacteria

E. coli XL1-blue cells (Strategene, La Jolla, CA) which had been transformed with the expression plasmids pDISC3x19-Ll and pDISC3x19-SL, respectively, were cultured overnight in 2xYT medium with 50 μg/ml ampicillin and 100 mM glucose ($2xYT_{Ga}$) at 37°C. 1:50 dilutions of the overnight cultures in $2xYT_{GA}$ were cultured as flask cultures at 37°C while shaking with 200 rpm. When the cultures had reached an OD_{600} value of 0.8, the bacteria were pelleted by 10-minute centrifugation with 1500 g at 20°C and resuspended in the same volume of a fresh 2xYT medium containing 50 μg ampicillin and 0.4 M saccharose. IPTG was added up to a final concentration of 0.1 mM, and the growth was continued at room temperature (20-22°C) for 18 - 20 h. The cells were harvested by 10-minute centrifugation with 5000 g at 4°C. The culture supernatant was held back and stored on ice. In order to isolate the soluble periplasmic proteins, the pelleted bacteria were resuspended in 5 % of the initial volume of ice-cold 50 mM Tris_HCl, 20 % saccharose, 1 mM EDTA, pH 8.0. Following 1 hour of incubation on ice with occasional stirring the spheroplasts were centrifuged with 30,000 g at 4°C for 30 minutes, the soluble periplasmic extract being obtained as supernatant and the spheroplasts with the insoluble periplasmic material being obtained as pellet. The culture supernatant and the soluble periplasmic extract were combined and clarified by further centrifugation (30,000 g, 4°C, 40 min.). The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70 % saturation). The protein precipitate was obtained by centrifugation (10,000 g, 4°C, 40 min.) and dissolved in 10 % of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. An immobilized metal affinity chromatography (IMAC) was carried out at 4°C using a 5 ml column of chelating sepharose (Pharmacia) which was charged with Cu^{2+} and had been equilibrated with 50 mM Tris-HC1, 1 M NaCl, pH 7.0 (starting buffer). The sample was loaded by passing it over the column. It was then washed with twenty column volume of starting buffer, followed by starting buffer with 50 mM imidazole until the

absorption at 280 nm of the effluent was at a minimum (about thirty column volumes). The absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0.

The protein concentrations were determined with the Bradford dye binding test [(](Bradford, 1976, *Anal. Biochem.* 72[,]:248-254) using the Bio-Rad (Munich, Germany) protein assay kit. The concentrations of the purified tetravalent and bivalent F_v antibody constructs were determined from the A_{280} values using the extinction coefficients $\epsilon^{1mg/ml} = 1.96$ and 1.93, respectively.

D. **Example 4: Expression of the [tetravalent and/or bivalent antibody construct in the yeast Pichia pastoris] Tetravalent And/or Bivalent Antibody Construct in the Yeast *Pichia Pastoris***

Competent *P. pastoris* GS155 cells (Invitrogen) were electroporated in the presence of 10 μg plasmid-DNA of pPIC-DISC-LL and pPIC-DISC-SL, respectively, which had been linearized with SacI. The transformants were selected for 3 days at 30°C on YPD plates containing 100 μg Zeocin™. The clones which secreted the bivalent and/or tetravalent F_v antibody constructs were selected by plate screening using an anti-c-myc-mAK 9E10 (IC chemikalien, Ismaning, Germany).

For the expression of the bivalent F_v antibody constructs and tetravalent F_v antibody constructs, respectively, the clones were cultured in YPD medium in shaking flasks for 2 days at 30°C with stirring. The cells were centrifuged resuspended in the same volume of the medium containing methanol and incubated for another 3 days at 30°C with stirring. The supernatants were obtained after the centrifguation. The recombinant product was isolated by ammonium sulfate precipitation, followed by IMAC as described above.

[Example]

E. **Examples 5: Characterization of the [tetravalent Fv antibody construct and bivalent Fv antibody construct, respectively,] Tetravalent F_v Antibody Construct and Bivalent F_v Antibody Construct, Respectively**

(A) Size exclusion chromatography

An analytical gel filtration of the F_v antibody constructs was carried out in PBS using a superdex 200-HR10/30 column (Pharmacia). The sample volume and the flow rate

were 200 μl/min and 0.5 ml/min, respectively. The column was calibrated with high-molecular and low-molecular gel filtration calibration kits (Pharmacia).

(B) Flow cytometry

The human $CD3^+/CD19^-$ -acute T-cell leukemia line Jurkat and the $CD19^+/CD3^-$ B-cell line JOK-1 were used for flow cytometrie. 5 x 10^5 cells in 50 μl RPMI 1640 medium (GIBCO BRL, Eggestein, Germany) which was supplemented with 10 % FCS and 0.1 % sodium azide (referred to as complete medium) were incubated with 100 μl of the F_v antibody preparations for 45 minute on ice. After washing using the complete medium the cells were incubated with 100 μl 10 μg/ml anti-c-myc-Mak 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second wash cycle, the cells were incubated with 100 μl of the FITC-labeled goat-anti-mouse-IgG (GIBCO BRL) under the same conditions as before. The cells were then washed again and resuspended in 100 μl 1 μg/ml propidium iodide solution (Sigma, Deisenhofen, Germany) in complete medium with the exclusion of dead cells. The relative fluorescence of the stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

(C) Cytotoxicity text

The CD19-expressing Burkitt lymphoma cell line Raji and Namalwa were used as target cells. The cells were incubated in RPMI (GIBCO BRL) which was supplemented with 10 % heat-inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate, at 37°C in a dampened atmosphere with 7.5 % CO_2. The cytotoxic T-cell tests were carried out in RPMI-1640 medium supplemented with 10 % FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. The cytotoxic activity was evaluated using a standard [^{51}Cr] release test; 2 x 10^6 target cells were labeled with 200 μCi Na[^{51}Cr]O_4 (Amersham-Buchler, Braunschweig, Germany) and washed 4 times and then resuspended in medium in a concentration of 5 x 10^6/ml. Increasing amounts of CTLs in 100 μl were titrated to 10^4 target cells/well or cavity in 50 μl. 50 μl antibodies were added to each well. The entire test was prepared three times and incubated at 37°C for 4 h. 100 μl of the supernatant were collected and tested for [^{51}Cr] release in a gamma counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). The maximum release was determined by

incubation of the target cells in 10 % SDS, and the spontaneous release was determined by incubation of the cells in medium alone. The specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100.

[Example]

F. Examples 6: Construction of the [plasmids pDISC5-LL and pDISC5-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific Fv antibody constructs in bacteria by high cell density fermentation] Plasmids Pdisc5-ll and Pdisc5-sl for the Expression of Bivalent, Bispecific And/or Tetravalent, Bispecific F_v Antibody Constructs in Bacteria by High Cell Density Fermentation

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the skp/OmpH periplasmic factor for a greater production of recombinant antibodies. The skp gene was amplified by PCR using the primers skp-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G and skp-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G using the plasmid pGAH317 (Holck and Kleppe, 1988, *Gene* 67[,]:117-124). The resulting PCR fragment was cleaved by AflII and HindIII and inserted in the AflII/HindIII-linearized plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers fe-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC and fe-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G. The XbaI/AflII-cleaved PCR fragments were inserted in pSKK before the skp insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the lac promoter/operator system [(cf. figs. 9, 10).](FIGURES 9 and 10).

[Claims]

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A multivalent F_v antibody construct having at least four variable domains which are linked with one another via the peptide linkers 1, 2 and 3.

2. The F_v antibody construct according to claim 1, wherein the peptide linkers 1 and 3 have 0 to 10 amino acids.

3. The F_v antibody construct according to claim 2, wherein the peptide linkers 1 and 3 have the amino acid sequence GG.

4. The F_v antibody construct according to any of claims 1 to 3, wherein the F_v antibody construct is bivalent.

5. The F_v antibody construct according to claim 4, wherein the peptide linker 2 has 11 to 20 amino acids.

6. The F_v antibody construct according to claim 4 or 5, wherein the peptide linker 2 has the amino acid sequence $(G_4S)_4$.

7. The F_v antibody construct according to any of claims 1 to 3, wherein the F_v antibody construct is tetravalent.

8. The F_v antibody construct according to claim 7, wherein the peptide linker 2 has 3 to 10 amino acids

9. The F_v antibody construct according to claim 7 or 8, wherein the peptide linker 2 comprises the amino acid sequence GGPGS.

10. The F_v antibody construct according to any of claims 1 to 9, wherein the F_v antibody construct is multispecific.

11. F_v antibody construct according to claim 10, wherein the F_v antibody construct is bispecific.

12. The F_v antibody construct according to any of claims 1 to 9, wherein the F_v antibody construct is monospecific.

13. A method of producing the multivalent F_v antibody construct according to any of claims 1 to 12, wherein DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid.

14. Expression plasmid coding for the multivalent F_v antibody construct according to any of claims 1 to 12.

15. The expression plasmid according to claim 14, namely pDISC3x19-LL.

16. The expression plasmid according to claim 14, namely pDISC3x19-SL.

17. The expression plasmid according to claim 14, namely pPIC-DISC-LL.

18. The expression plasmid according to claim 14, namely pPIC-DISC-SL.

19. The expression plasmid according to claim 14, namely pDISC5-LL.

20. The expression plasmid according to claim 14, namely pDISC5-SL.

21. Use of the multivalent F_v antibody construct according to any of claims 1 to 12 for the diagnosis and/or treatment of diseases.

22. Use according to claim 21, wherein he diseases are viral, bacterial or tumoral diseases.

Claims As Amended In Response To Written Opinion

1. A multivalent F_V antibody construct having at least four variable domains which are linked with one another via the peptide linkers 1, 2 and 3, wherein the peptide linkers 1 and 3 have 0 to 10 amino acids.

2. The F_V antibody construct according to claim 1, wherein the peptide linkers 1 and 3 have the amino acid sequence GG.

3. The F_V antibody construct according to claim 1 or 2, wherein the F_V antibody construct is bivalent.

4. The F_V antibody construct according to claim 3, wherein the peptide linker 2 has 11 to 20 amino acids.

5. The F_V antibody construct according to claim 3 or 4, wherein the peptide linker 2 has the amino acid sequence $(G_4S)_4$.

6. The F_V antibody construct according to claim 1 or 2, wherein the F_V antibody construct is tetravalent.

7. The F_V antibody construct according to claim 6, wherein the peptide linker 2 has 3 to 10 amino acids.

8. The F_V antibody construct according to claim 6 or 7, wherein the peptide linker 2 comprises the amino acid sequence GGPGS.

9. The F_V antibody construct according to any of claims 1 to 8, wherein the F_V antibody construct is multispecific.

10. F_V antibody construct according to claim 9, wherein the F_V antibody construct is bispecific.

11. The F_V antibody construct according to any of claims 1 to 8, wherein the F_V antibody construct is monospecific.

12. A method of producing the multivalent F_V antibody construct according to any of claims 1 to 11, wherein DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_V antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid.

13. Expression plasmid coding for the multivalent F_V antibody construct according to any of claims 1 to 11.

14. The expression plasmid according to claim 13, namely pDISC3x19-LL.

15. The expression plasmid according to claim 13, namely pDISC3x19-SL.

16. The expression plasmid according to claim 13, namely pPIC-DISC-LL.

17. The expression plasmid according to claim 13, namely pPIC-DISC-SL.

18. The expression plasmid according to claim 13, namely pDISC5-LL.

19. The expression plasmid according to claim 13, namely pDISC6-SL.

20. Use of the multivalent F_V antibody construct according to any of claims 1 to 11 for the diagnosis and/or treatment of diseases.

21. Use according to claim 20, wherein the diseases are viral, bacterial or tumoral diseases.

ABSTRACT [of the Disclosure]

The present invention relates to a multivalent F_v antibody construct having at least four variable domains which are linked with each over via the peptide linkers 1, 2 and 3. The invention also concerns expression plasmids which code for such an F_v antibody construct and a method of producing the F_v antibody constructs as well as their use.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:
LITTLE and KIPRIYANOV

Serial No.: 09/674,794 Group Art Unit: To be assigned

I.A. Filing Date: May 5, 1999 Examiner: To be assigned

For: *Multivalent Antibody Constructs* Attorney Docket No.: 8484-089-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notification of Missing Requirements mailed May 22, 2001, Applicants attorney, in connection with the above-identified patent application, submits herewith a Sequence Listing in computer readable form in compliance with 37 C.F.R. §§ 1.821(e).

I hereby state that the content of the paper copy of the Sequence Listing submitted on November 3, 2000 and the computer readable copy of the Sequence Listing submitted herewith, in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, are the same.

In accordance with the Rules of Practice, please enter the following amendments and consider the remarks below prior to the examination of the above-captioned application.

IN THE SPECIFICATION

On page 8, please replace the paragraph beginning, "The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma..." with the following paragraph:

The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov *et al.*, 1996, *J.-Immunol. Meth.* 196:51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov *et al.*, 1997, *Protein Eng.* 10:445-453), respectively, were used for the construction of expression plasmids for a single-chain F_v antibody construct. A PCR

fragment 1 of the V_H domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-TTAGATCTATTAAAGAGGAGAAATTAACC (SEQ ID NO:1) and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC (SEQ ID NO:2) (FIGURE. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR fragment 2 of the V_L domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidinyl tail, was produced using the primers DP3, 5;-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA, (SEQ ID NO:3) and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-GGTGATGTGAGTT TAGG (SEQ ID NO:4). The PCR fragment 2 was cleaved by HindIII and XbaI and ligated with the HindIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 (FIGURE 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG (SEQ ID NO:5) and either Li-1, 5'-TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-CCACCACCGC TACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATCAGCCCG,(SEQ ID NO:6) for the production of a long flexible $(Gly_4Ser)_4$ inter-scFV linker (PCR fragment 3, FIGURE 2) or Li-2, 5'-TATATA-CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATCA GCCG, (SEQ ID NO:7) for the production of a short rigid GGPGS linker (PCR fragment 4, FIGURE 2). The expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the NcoI/PvuII restriction fragment from pHOG19-3, comprising the vector framework and the NcoI/PVuII-cleaved PCR fragments 3 and 4, respectively (FIGURES 3 and 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F_v antibody constructs are indicated in FIGURES 5 and 6, respectively.

On page 9, please replace the paragraph beginning, "The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins..." with the following paragraph:

The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal,

followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli.* The gene which codes for the tetravalent F_v antibody construct (scDia-SL) was amplified by means of PCR by the template pDIC3x19-SL using the primers 5-PIC, 5'-CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn (SEQ ID NO:8). 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG (SEQ ID NO:9). The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences of the tetravalent F_v antibody construct are shown in FIGURE 7.

On page 13, please replace the paragraph beginning, "Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system..." with the following paragraph:

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the skp/OmpH periplasmic factor for a greater production of recombinant antibodies. The skp gene was amplified by PCR using the primers skp-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G (SEQ ID NO:10) and skp-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G (SEQ ID NO:11) using the plasmid pGAH317 (Holck and Kleppe, 1988, *Gene* 67:117-124). The resulting PCR fragment was cleaved by Afl II and HindIII and inserted in the Afl II/HindIII-linearized plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers fe-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC (SEQ ID NO:12) and fe-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G (SEQ ID NO:13). The XbaI/AflII-cleaved PCR fragments were inserted in pSKK before the skp insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the lac promoter/operator system (FIGURES 9 and 10).

REMARKS

The specification has been amended to incorporate the SEQ ID NOS of the various sequences disclosed therein by their respective SEQ ID NOS as assigned in the Sequence Listing being submitted concurrently herewith. No new matter is introduced by virtue of these amendments. Accordingly, Applicants kindly request that they be entered into the instant application.

The Commissioner is hereby authorized to charge any required fee or credit any overpayment to Pennie & Edmonds LLP Deposit Account no. 16-1150. This page is submitted in duplicate for such purpose.

Respectfully submitted,

Dated: August 21, 2001

Birgit Millauer 43,341 (Reg. No.)

Laura A. Coruzzi

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REMARKS

The specification has been amended to incorporate the SEQ ID NOS of the various sequences disclosed therein by their respective SEQ ID NOS as assigned in the Sequence Listing being submitted concurrently herewith. No new matter is introduced by virtue of these amendments. Accordingly, Applicants kindly request that they be entered into the instant application.

The Commissioner is hereby authorized to charge any required fee or credit any overpayment to Pennie & Edmonds LLP Deposit Account no. 16-1150. This page is submitted in duplicate for such purpose.

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Respectfully submitted,

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Exhibit A
Marked Up Versions of Amended Paragraphs
(Additions are italicized, deletions are bracketed)

Amended paragraph on page 8, beginning, "The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma...":

The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov *et al.*, 1996, *J.-Immunol. Meth.* 196:51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov *et al.*, 1997, *Protein Eng.* 10:445-453), respectively, were used for the construction of expression plasmids for a single-chain F_v antibody construct. A PCR fragment 1 of the V_H domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-TTAGATCTATTAAAGAGGAGAAATTAACC *(SEQ ID NO:1)* and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC *(SEQ ID NO:2)* FIGURE. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR fragment 2 of the V_L domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidinyl tail, was produced using the primers DP3, 5'-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA *(SEQ ID NO:3)* and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-GGTGATGTGAGTTTAGG *(SEQ ID NO:4)*. The PCR fragment 2 was cleaved by HindIII and XbaI and ligated with the HindIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 (FIGURE 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG *(SEQ ID NO:5)* and either Li-1, 5'-TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-CCACCACC GCTACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATCAGCCCG *(SEQ ID NO:6)* for the production of a long flexible $(Gly_4Ser)_4$ inter-scFV linker (PCR fragment 3, FIGURE 2) or Li-2, 5'-TATATA-CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATC

AGCCG, *(SEQ ID NO:7)* for the production of a short rigid GGPGS linker (PCR fragment 4, FIGURE 2). The expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the NcoI/PvuII restriction fragment from pHOG19-3, comprising the vector framework and the NcoI/PVuII-cleaved PCR fragments 3 and 4, respectively (FIGURES 3 and 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F_v antibody constructs are indicated in FIGURES 5 and 6, respectively.

Amended paragraph on page 9, beginning, "The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins..."

The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal, followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli*. The gene which codes for the tetravalent F_v antibody construct (scDia-SL) was amplified by means of PCR by the template pDIC3x19-SL using the primers 5-PIC, 5'-CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn *(SEQ ID NO:8)*. 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG *(SEQ ID NO:9)*. The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences of the tetravalent F_v antibody construct are shown in FIGURE 7.

Amended paragraph on page 13, beginning, "Expression vectors were prepared which contained the hok/sok plasmid-free cell..."

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the skp/OmpH periplasmic factor for a greater production of recombinant antibodies. The skp gene was amplified by PCR using the primers skp-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G *(SEQ ID NO:10)* and skp-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G *(SEQ ID NO:11)* using the plasmid pGAH317 (Holck and Kleppe, 1988, *Gene* 67:117-124). The resulting PCR fragment was cleaved by

Afl II and HindIII and inserted in the Afl II/HindIII-linearized plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers fe-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC *(SEQ ID NO:12)* and fe-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G *(SEQ ID NO:13)*. The XbaI/AflII-cleaved PCR fragments were inserted in pSKK before the skp insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the lac promoter/operator system (FIGURES 9 and 10).

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Multivalent Antibody Constructs

The present invention relates to multivalent F_v antibody constructs, expression plasmids which code for them, and a method for producing the F_v antibody constructs as well as the use thereof.

Natural antibodies are dimers and are therefore referred to as bivalent. They have four variable domains, namely two V_H domains and two V_L domains. The variable domains serve as binding sites for an antigen, a binding site being formed from a V_H domain and a V_L domain. Natural antibodies recognize one antigen each, so that they are also referred to as monospecific. Furthermore, they also have constant domains which add to the stability of the natural antibodies. On the other hand, they are also co-responsible for undesired immune responses which result when natural antibodies of various animal species are administered mutually.

In order to avoid such immune responses, antibodies are constructed which lack the constant domains. In particular, these are antibodies which only comprise the variable domains. Such antibodies are designated F_v antibody constructs. They are often available in the form of single-chain monomers paired with one another.

However, it showed that F_v antibody constructs only have little stability. Therefore, their usability for therapeutic purposes is strongly limited.

Thus, it is the object of the present invention to provide an antibody by means of which undesired immune responses can be avoided. Furthermore, it shall have a stability which makes it usable for therapeutic uses.

According to the invention this is achieved by the subject matters defined in the claims.

Therefore, the subject matter of the present invention relates to a multivalent F_v antibody construct which has great stability. Such a construct is suitable for diagnostic and therapeutic purposes.

The present invention is based on the applicant's insights that the stability of an F_v antibody construct can be increased if it is present in the form of a single-chain dimer where the four variable domains are linked with one another via three peptide linkers. The applicant also recognized that the F_v antibody construct folds with itself when the middle peptide linker has a length of about 10 to 30 amino acids. The applicant also recognized that the F_v antibody construct folds with other F_v antibody constructs when the middle peptide linker has a length of about up to 10 amino acids so as to obtain a multimeric, i.e. multivalent, F_v antibody construct. The applicant also realized that the F_v antibody construct can be multi-specific.

According to the invention the applicant's insights are utilized to provide a multi-valent F_v antibody construct

which comprises at least four variable domains which are linked with one another via peptide linkers 1, 2 and 3.

The expression "F_v antibody construct" refers to an antibody which has variable domains but no constant domains.

The expression "multivalent F_v antibody construct" refers to an F_v antibody which has several, but at least four, variable domains. This is achieved when the single-chain F_v antibody construct folds with itself so as to give four variable domains, or folds with other single-chain F_v antibody constructs. In the latter case, an F_v antibody construct is given which has 8, 12, 16, etc., variable domains. It is favorable for the F_v antibody construct to have four or eight variable domains, i.e. it is bivalent or tetravalent (cf. Fig. 1). Furthermore, the variable domains may be equal or differ from one another, so that the antibody construct recognizes one or several antigens. The antibody construct preferably recognizes one or two antigens, i.e. it is monospecific and bispecific, respectively. Examples of such antigens are proteins CD19 and CD3.

The expression "peptide linkers 1, 3" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linkers 1 and 3 may be equal or differ from each other. Furthermore, the peptide linker may have a length of about 0 to 10 amino acids. In the former case, the peptide linker is only a peptide bond from the COOH residue of one of the variable domains and the NH_2 residue of another of the variable domains. The peptide linker preferably comprises the amino acid sequence GG.

The expression "peptide linker 2" refers to a peptide linker adapted to link variable domains of an F_v antibody construct with one another. The peptide linker may contain any amino acids, the amino acids glycine (G), serine (S) and proline (P) being preferred. The peptide linker may also have a length of about 3 to 10 amino acids, in partiuclar 5 amino acids, and most particularly the amino acid sequence GGPGS, which serves for achieving that the single-chain F_v antibody construct folds with other single-chain F_v antibody constructs. The peptide linker can also have a length of about 11 to 20 amino acids, in particular 15 to 20 amino acids, and most particularly the amino acid sequence $(G_4S)_4$, which serves for achieving that the single-chain F_v antibody construct folds with itself.

An F_v antibody construct according to the invention can be produced by common methods. A method is favorable in which DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAs coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid. Reference is made to Examples 1 to 6. As to the expressions "F_v antibody construct" and "peptide linker" reference is made to the above explanations and, by way of supplement, to Maniatis, T. et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory 1982.

DNAs which code for an F_v antibody construct according to the invention also represent a subject matter of the present invention. Furthermore, expression plasmids which contain such DNAs also represent a subject matter of the present invention. Preferred expression plasmids are pDISC3x19-LL,

pDISC3x19-SL, pPIC-DISC-LL, pPIC-DISC-SL, pDISC5-LL and pDISC6-SL. The first four were deposited with the DSMZ (*Deutsche Sammlung für Mikroorganismen und Zellen)* [German-type collection for micro-organisms and cells] on April 30, 1998 under DSM 12150, DSM 12149, DSM 12152 and DSM 12151, respectively.

Another subject matter of the present invention relates to a kit, comprising:

(a) an F_v antibody construct according to the invention, and/or
(b) an expression plasmid according to the invention, and
(c) conventional auxiliary agents, such as buffers, solvents and controls.

One or several representatives of the individual components may be present.

The present invention provides a multivalent F_v antibody construct where the variable domains are linked with one another via peptide linkers. Such an antibody construct distinguishes itself in that it contains no parts which can lead to undesired immune reactions. Furthermore, it has great stability. It also enables to bind several antigens simultaneously. Therefore, the F_v antibody construct according to the invention is perfectly adapted to be used not only for diagnostic but also for therapeutic purposes. Such purposes can be seen as regards any disease, in particular a viral, bacterial or tumoral disease.

Brief description of the drawings:

Fig. 1 shows the genetic organization of an F_v antibody construct (A) according to the invention and schemes for forming a bivalent (B) or tetravalent F_v antibody construct (C). Ag: antigen; His_6: six C-terminal histidine residues; stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

Fig. 2 shows the scheme for the construction of the plasmids pDISC3x19-LL and pDISC3x19-SL. c-myc: sequence coding for an epitope which is recognized by the antibody 9E1, His_6: sequence which codes for six C-terminal histidine residues; PelB: signal peptide sequence of the bacterial pectate lyase (PelB leader); rbs: ribosome binding site; Stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chains.

Fig. 3 shows a diagram of the expression plasmid pDISC3x19-LL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; ColE1: origin of the DNA replication; f1-IG: intergenic region of the bacteriophage f1; Lac P/O: wt lac-operon promoter/operator; linker 1: sequence which codes for a GlyGly dipeptide linking the V_H and V_L domains; linker 2: sequence coding for a $(Gly_4Ser)_4$ polypeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

Fig. 4 shows a diagram of the expression plasmid pDISC3x19-SL. 6xHis: sequence which codes for six C-terminal histidine

residues; bla: gene which codes for ß-lactamase which is responsible for the ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope recognized by the 9E10 antibody; ColE1: origin of DNA replication; f1-IG: intergenic region of the bacteriophage f1; Lac P/O: wt lac-operon promoter/operator: linker 1: sequence which codes for a GlyGly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide which links the hybrid scFv fragments; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

Fig. 5 shows the nucleotide sequence and the amino acid sequence derived therefrom of the bivalent F_v antibody construct encoded by the expression plasmid pDIS3x19-LL. c-myc epitope: sequence coding for an epitope which is recognized by the antibody 9E10; CDR: region determining the complementarity; framework: framework region; His6 tail: sequence which codes for six C-terminal histidine residues; PelB leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

Fig. 6 shows the nucleotide sequence and the derived amino acid sequence of the tetravalent F_v antibody construct encoded by the expression plasmid pDISC3x19-SL. c-myc epitope: sequence coding for an epitope which is recognized by the 9E10 antibody; CDR: region determining complementarity; framework: framework region; His6 tail: sequence coding for the six C-terminal histidine residues; PelB leader: signal peptide sequence of the bacterial pectate lyase; RBS: ribosome binding site; V_H and V_L: variable region of the heavy and light chains.

Fig. 7 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene which codes for an α-factor leader sequence and a gene coding for the tetravalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-SL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae*-α factor secretion signal; V_H: variable region of the heavy chain. Rhombs indicate the signal cleaving sites.

Fig. 8 shows the nucleotide sequence and the derived amino acid sequence of a connection between a gene coding for an α-factor leader sequence and a gene which codes for the bivalent F_v antibody construct in the *Pichia* expression plasmid pPIC-DISC-LL. Alpha-factor signal: leader peptide sequence of the *Saccharomyces cerevisiae*-α factor secretion signal; V_H: variable region of the heavy chain. Rhombs show the signal cleaving sites.

Fig. 9 shows a diagram of the expression plasmid pDISC5-LL. 6xHis: sequence coding for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs; c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilizing DNA locus; LacI: gene which codes for the Lac repressor; Lac P/O: wt lac-operon-promoter/operator; LacZ': gene which codes for the α-peptide of ß-galactosidase; linker 1: sequence which codes for a GlyGly dipeptide connecting the V_H and V_L domains; linker 2: sequence which codes for a $(Gly_4Ser)_4$ polypeptide linking the hybrid scFv fragments; M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site which originates

from the E. coli lacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; V_H and V_L: variable region of the heavy and light chains.

Fig. 10 shows a diagram of the expression plasmid pDISC6-SL. 6xHis: sequence which codes for six C-terminal histidine residues; bla: gene which codes for ß-lactamase responsible for ampicillin resistance; bp: base pairs: c-myc: sequence coding for an epitope which is recognized by the 9E10 antibody; hok-sok: plasmid-stabilized DNA locus; LacI: gene which codes for the Lac repressor; Lac P/O: wt lac-operon promoter/operator; LacZ': gene which codes for the α-peptide of ß-galactosidase; linker 1: sequence which codes for a GlyGly dipeptide which links the V_H and V_L domains; linker 3: sequence which codes for a GlyGlyProGlySer oligopeptide linking the hybrid scFv fragments: M13 IG: intergenic region of the M13 bacteriophage; pBR322ori: origin of DNA replication; Pel-B leader: signal peptide sequence of the bacterial pectate lyase; rbs: ribosome binding site originating from the *E. coli* lacZ gene (lacZ), from the bacteriophage T7 gene 10 (T7g10) or from the *E. coli* skp gene (skp); skp: gene which codes for the bacterial periplasmic factor Skp/OmpH; tHP: strong transcription terminator; tIPP: transcription terminator; V_H and V_L: variable region of the heavy and light chains.

The invention is explained by the below examples.

Example 1: Construction of the plasmids pDISC3x19-LL and pDISC3x19-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific F_V antibody constructs in bacteria

The plasmids pHOG-αCD19 and pHOG-dmOKT3 which code for the scFv fragments derived from the hybridoma HD37 which is specific to human CD19 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62) and from the hybridoma OKT3 which is specific to human CD3 (Kipriyanov et al., 1997, Protein Eng. 10, 445-453), respectively, were used for the construction of expression plasmids for a single-chain F_V antibody construct. A PCR fragment 1 of the V_H domain of anti-CD19, followed by a segment which codes for a GlyGly linker, was produced using the primers DP1, 5'-TCACACAGAATTC-TTAGATCTATTAAAGAGGAGAAATTAACC, and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC (cf. Fig. 2). The PCR fragment 1 was cleaved by EcoRI and EcoRV and ligated with the EcoRI/EcoRV-linearized plasmid pHOG-dmOKT3 so as to produce the vector pHOG19-3. The PCR fragment 2 of the V_L domain of anti-CD19, followed by a segment which codes for a c-myc epitope and a hexahistidinyl tail, was produced using the primers DP3, 5'-AGCACACAAGCTTGGCGGTGATATCTTGCTCACCCAAAC-TCCA, and DP4, 5'-AGCACACTCTAGAGACACACAGATCTTTAGTGATGGTGAT-GGTGATGTGAGTTTAGG. The PCR fragment 2 was cleaved by HindIII and XbaI and ligated with the HIndIII/XbaI-linearized plasmid pHOG-dmOKT3 so as to obtain the vector pHOG3-19 (cf. Fig. 2). The gene coding for the hybrid scFv-3-19 in the plasmid pHOG3-19 was amplified by means of PCR with the primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG and either Li-1, 5'-TATATACTGCAGCTGCACCTGGCTACCACCACCACCGGAGCCG-CCACCACCGCTACCACCGCCGCCAGAACCACCACCACCAGCGGCCGCAGCATCAGCCCG, for the production of a long flexible $(Gly_4Ser)_4$ inter-scFV linker (PCR fragment 3, cf. Fig. 2) or Li-2, 5'-TATATA-

CTGCAGCTGCACCTGCGACCCTGGGCCACCAGCGGCCGCAGCATCAGCCCG, for the production of a short rigid GGPGS linker (PCR fragment 4, cf. Fig. 2). The expression plasmids pDISC3x19-LL and pDISC3x19-SL were constructed by ligating the NcoI/PvuII restriction fragment from pHOG19-3, comprising the vector framework and the NcoI/PvuII-cleaved PCR fragments 3 and 4, respectively (cf. Figs. 3, 4). The complete nucleotide and protein sequences of the bivalent and tetravalent F_v antibody constructs are indicated in Figs 5 and 6, respectively.

Example 2: Construction of the plasmids pPIC-DISC-LL and pPIC-DISC-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific F_v antibody constructs in yeast

(A) Construction of pPIC-DISC-SL

The vector pPICZαA (Invitrogen BV, Leek, Netherlands) for the expression and secretion of recombinant proteins in the yeast *Pichia pastoris* was used as a starting material. It contains a gene which codes for the *Saccharomyces cerevisiae* α-factor secretion signal, followed by a polylinker. The secretion of this vector is based on the dominant selectable marker, Zeocin™ which is bifunctional in both *Pichia* and *E. coli*. The gene which codes for the tetravalent F_v antibody construct (scDia-SL) was amplified by means of PCR by the template pDISC3x19-SL using the primers 5-PIC, 5'-CCGTGAATTCCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGC, and pSEXBn 5'-GGTCGACGTTAACCGACAAACAACAGATAAAACG. The resulting PCR product was cleaved by EcoRI and XbaI and ligated in EcoRI/XbaI-linearized pPICZαA. The expression plasmid pPIC-DISC-SL was obtained. The nucleotide and protein sequences

of the tetravalent F_v antibody construct are shown in Fig. 7.

(B) Construction of pPIC-DISC-LL

The construction of pPIC-DISC-LL was carried out on the basis of pPICZαA (Invitrogen BV, Leek, Netherlands) and pDISC3x19-LL (cf. Fig. 3). The plasmid-DNA pPICZαA was cleaved by EcoRI. The overhanging 5'-ends were filled using a Klenow fragment of the *E. coli* DNA polymerase I. The resulting DNA was cleaved by XbaI, and the large fragment comprising the pPIC vector was isolated. Analogous thereto the DNA of pDISC3x19-LL was cleaved by NcoI and treated with a Klenow fragment. Following the cleavage using XbaI a small fragment, comprising a gene coding for the bivalent F_v antibody, was isolated. Its ligation with a pPIC-derived vector-DNA resulted in the plasmid pPIC-DISC-LL. The nucleotide and protein sequences of the bivalent F_v antibody construct are shown in Fig. 8.

Example 3: Expression of the tetravalent and/or bivalent F_v antibody construct in bacteria

E. coli XL1-blue cells (Strategene, La Jolla, CA) which had been transformed with the expression plasmids pDISC3x19-LL and pDISC3x19-SL, respectively, were cultured overnight in 2xYT medium with 50 µg/ml ampicillin and 100 mM glucose ($2xYT_{Ga}$) at 37°C. 1:50 dilutions of the overnight cultures in $2xYT_{GA}$ were cultured as flask cultures at 37°C while shaking with 200 rpm. When the cultures had reached an OD_{600} value of 0.8, the bacteria were pelleted by 10-minute centrifugation with 1500 g at 20°C and resuspended in the same volume of a fresh 2xYT medium containing 50 µg/ml ampicillin and 0.4 M saccharose. IPTG was added up to a

final concentration of 0.1 mM, and the growth was continued at room temperature (20-22°C) for 18 - 20 h. The cells were harvested by 10-minute centrifugation with 5000 g at 4°C. The culture supernatant was held back and stored on ice. In order to isolate the soluble periplasmic proteins, the pelleted bacteria were resuspended in 5 % of the initial volume of ice-cold 50 mM Tris-HCl, 20 % saccharose, 1 mM EDTA, pH 8.0. Following 1 hour of incubation on ice with occasional stirring the spheroplasts were centrifuged with 30,000 g at 4°C for 30 minutes, the soluble periplasmic extract being obtained as supernatant and the spheroplasts with the insoluble periplasmic material being obtained as pellet. The culture supernatant and the soluble periplasmic extract were combined and clarified by further centrifugation (30,000 g, 4°C, 40 min.). The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70 % saturation). The protein precipitate was obtained by centrifugation (10,000 g, 4°C, 40 min.) and dissolved in 10 % of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. An immobilized metal affinity chromatography (IMAC) was carried out at 4°C using a 5 ml column of chelating sepharose (Pharmacia) which was charged with Cu^{2+} and had been equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (starting buffer). The sample was loaded by passing it over the column. It was then washed with twenty column volumes of starting buffer, followed by starting buffer with 50 mM imidazole until the absorption at 280 nm of the effluent was at a minimum (about thirty column volumes). The absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0.

The protein concentrations were determined with the Bradford dye binding test (1976, Anal. Biochem. 72, 248-254) using the Bio-Rad (Munich, Germany) protein assay kit. The

concentrations of the purified tetravalent and bivalent F_v antibody constructs were determined from the A_{280} values using the extinction coefficients $\varepsilon^{1mg/ml}$ = 1.96 and 1.93, respectively.

Example 4: Expression of the tetravalent and/or bivalent antibody construct in the yeast *Pichia pastoris*

Competent *P. pastoris* GS155 cells (Invitrogen) were electroporated in the presence of 10 µg plasmid-DNA of pPIC-DISC-LL and pPIC-DISC-SL, respectively, which had been linearized with SacI. The transformants were selected for 3 days at 30°C on YPD plates containing 100 µg/ml Zeocin™. The clones which secreted the bivalent and/or tetravalent F_v antibody constructs were selected by plate screening using an anti-c-myc-mAk 9E10 (IC Chemikalien, Ismaning, Germany).

For the expression of the bivalent F_v antibody constructs and tetravalent F_v antibody constructs, respectively, the clones were cultured in YPD medium in shaking flasks for 2 days at 30°C with stirring. The cells were centrifuged resuspended in the same volume of the medium containing methanol and incubated for another 3 days at 30°C with stirring. The supernatants were obtained after the centrifugation. The recombinant product was isolated by ammonium sulfate precipitation, followed by IMAC as described above.

Example 5: Characterization of the tetravalent F_v antibody construct and bivalent F_v antibody construct, respectively,

(A) Size exclusion chromatography

An analytical gel filtration of the F_v antibody constructs was carried out in PBS using a superdex 200-HR10/30 column (Pharmacia). The sample volume and the flow rate were 200 µl/min and 0.5 ml/min, respectively. The column was calibrated with high-molecular and low-molecular gel filtration calibration kits (Pharmacia).

(B) Flow cytometry

The human $CD3^+/CD19^-$-acute T-cell leukemia line Jurkat and the $CD19^+/CD3^-$ B-cell line JOK-1 were used for flow cytometrie. 5 x 10^5 cells in 50 µl RPMI 1640 medium (GIBCO BRL, Eggestein, Germany) which was supplemented with 10 % FCS and 0.1 % sodium azide (referred to as complete medium) were incubated with 100 µl of the F_v antibody preparations for 45 minutes on ice. After washing using the complete medium the cells were incubated with 100 µl 10 µg/ml anti-c-myc-Mak 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second wash cycle, the cells were incubated with 100 µl of the FITC-labeled goat-anti-mouse-IgG (GIBCO BRL) under the same conditions as before. The cells were then washed again and resuspended in 100 µl 1 µg/ml propidium iodide solution (Sigma, Deisenhofen, Germany) in complete medium with the exclusion of dead cells. The relative fluorescence of the stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

(C) Cytotoxicity test

The CD19-expressing Burkitt lymphoma cell line Raji and Namalwa were used as target cells. The cells were incubated in RPMI 1640 (GIBCO BRL) which was supplemented with 10 %

heat-inactivated FCS (GIBCO BRL), 2 mM glutamine and 1 mM pyruvate, at 37°C in a dampened atmosphere with 7.5 % CO_2. The cytotoxic T-cell tests were carried out in RPMI-1640 medium supplemented with 10 % FCS, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate and 0.05 mM 2-ME. The cytotoxic activity was evaluated using a standard[^{51}Cr] release test; 2 x 10^6 target cells were labeled with 200 µCi Na[^{51}Cr]O_4 (Amersham-Buchler, Braunschweig, Germany) and washed 4 times and then resuspended in medium in a concentration of 2 x 10^5/ml. The effector cells were adjusted to a concentration of 5 x 10^6/ml. Increasing amounts of CTLs in 100 µl were titrated to 10^4 target cells/well or cavity in 50 µl. 50 µl antibodies were added to each well. The entire test was prepared three times and incubated at 37°C for 4 h. 100 µl of the supernatant were collected and tested for [^{51}Cr] release in a gamma counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). The maximum release was determined by incubation of the target cells in 10 % SDS, and the spontaneous release was determined by incubation of the cells in medium alone. The specific lysis (%) was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100.

Example 6: Construction of the plasmids pDISC5-LL and pDISC5-SL for the expression of bivalent, bispecific and/or tetravalent, bispecific F_v antibody constructs in bacteria by high cell density fermentation

Expression vectors were prepared which contained the hok/sok plasmid-free cell suicide system and a gene which codes for the Skp/OmpH periplasmic factor for a greater production of recombinant antibodies. The skp gene was amplified by PCR using the primers skp-1, 5'-CGA ATT CTT AAG ATA AGA AGG AGT

TTA TTG TGA AAA AGT GGT TAT TAG CTG CAG G and skp-2, 5'-CGA ATT AAG CTT CAT TAT TTA ACC TGT TTC AGT ACG TCG G using the plasmid pGAH317 (Holck and Kleppe, 1988, Gene 67, 117-124). The resulting PCR fragment was cleaved by AflII and HindIII and inserted in the AflII/HindIII-linearized plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) so as to obtain the vector pSKK. The genes obtained in the plasmids pDISC3x19-LL and pDISC3x19-SL and coding for the scFv antibody constructs were amplified by means of the primers fe-1, 5'-CGA ATT TCT AGA TAA GAA GGA GAA ATT AAC CAT GAA ATA CC and fe-2, 5'-CGA ATT CTT AAG CTA TTA GTG ATG GTG ATG GTG ATG TGA G. The XbaI/AflII-cleaved PCR fragments were inserted in pSKK before the skp insert so as to obtain the expression plasmids pDISC5-LL and pDISC6-SL, respectively, which contain tri-cistronic operons under the control of the lac promoter/operator system (cf. figs. 9, 10).

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Attorney's File: K 2675

Amended Claims

1. A multivalent F_v antibody construct having at least four variable domains which are linked with one another via the peptide linkers 1, 2 and 3, wherein the peptide linkers 1 and 3 have 0 to 10 amino acids.

2. The F_v antibody construct according to claim 1, wherein the peptide linkers 1 and 3 have the amino acid sequence GG.

3. The F_v antibody construct according to claim 1 or 2, wherein the F_v antibody construct is bivalent.

4. The F_v antibody construct according to claim 3, wherein the peptide linker 2 has 11 to 20 amino acids.

5. The F_v antibody construct according to claim 3 or 4, wherein the peptide linker 2 has the amino acid sequence $(G_4S)_4$.

6. The F_v antibody construct according to claim 1 or 2, wherein the F_v antibody construct is tetravalent.

7. The F_v antibody construct according to claim 6, wherein the peptide linker 2 has 3 to 10 amino acids.

8. The F_v antibody construct according to claim 6 or 7, wherein the peptide linker 2 comprises the amino acid sequence GGPGS.

9. The F_v antibody construct according to any of claims 1 to 8, wherein the F_v antibody construct is multispecific.

10. F_v antibody construct according to claim 9, wherein the F_v antibody construct is bispecific.

11. The F_v antibody construct according to any of claims 1 to 8, wherein the F_v antibody construct is monospecific.

12. A method of producing the multivalent F_v antibody construct according to any of claims 1 to 11, wherein DNAs coding for the peptide linkers 1, 2 and 3 are ligated with DNAS coding for the four variable domains of an F_v antibody construct such that the peptide linkers link the variable domains with one another and the resulting DNA molecule is expressed in an expression plasmid.

13. Expression plasmid coding for the multivalent F_v antibody construct according to any of claims 1 to 11.

14. The expression plasmid according to claim 13, namely pDISC3x19-LL.

15. The expression plasmid according to claim 13, namely pDISC3x19-SL.

16. The expression plasmid according to claim 13, namely pPIC-DISC-LL.

17. The expression plasmid according to claim 13, namely pPIC-DISC-SL.

18. The expression plasmid according to claim 13, namely pDISC5-LL.

19. The expression plasmid according to claim 13, namely pDISC6-SL.

20. Use of the multivalent F_v antibody construct according to any of claims 1 to 11 for the diagnosis and/or treatment of diseases.

21. Use according to claim 20, wherein the diseases are viral, bacterial or tumoral diseases.

Abstract of the Disclosure

The present invention relates to a multivalent F_v antibody construct having at least four variable domains which are linked with each over via the peptide linkers 1, 2 and 3. The invention also concerns expression plasmids which code for such an F_v antibody construct and a method of producing the F_v antibody constructs as well as their use.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Little *et al.*

Application No.: To be assigned

Group Art Unit: To be assigned

Filed:

Examiner: To be assigned

For: MULTIVALENT ANTIBODY CONSTRUCTS

Attorney Docket No.: 8484-089-999

POWER OF ATTORNEY BY ASSIGNEE
AND EXCLUSION OF INVENTOR(S) UNDER 37 C.F.R. 3.71

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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35399), Scott B. Familant (Reg. No. 35514), Kelly D. Talcott (Reg. No. 39582), Francis D. Cerrito (Reg. No.38100), Anthony M. Insogna (Reg. No. 35203), Brian M. Rothery (Reg. No. 35340), Brian D. Siff (Reg. No. 35679), and Alan Tenenbaum (Reg. No. 34939), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 3300 Hillview Avenue, Palo Alto, CA 94304, all of Pennie & Edmonds LLP (PTO Customer No. 20583), as its attorneys to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventors and their attorney(s) in accordance with the provisions of 37 C.F.R. 3.71, provided that, if any one of these attorneys ceases being affiliated with the law firm of Pennie & Edmonds LLP as partner, counsel, or employee, then the appointment of that attorney and all powers derived therefrom shall terminate on the date such attorney ceases being so affiliated.

An assignment of the entire interest in the above-identified subject application:

[ ] was recorded on ________________ at reel/frame _/______.

[X] is submitted herewith for recording.

Please direct all correspondence for this application to customer no. 20583.

ASSIGNEE:

Signature:

Typed Name: Prof. Dr. Harald zur Hausen | Dr. Josef Puchta

Position/Title: Scientific Member of the Management Board | Administrative Member of the Management Board

Address: Deutsches Krebsforschungszentrum Stiftung des Offentlichen Rechts
Im Neuenheimer Feld 280 69120 Heidelberg
Germany

Date: 05. Dez. 00

CA1 - 259033.1

K.2839

10 Rec'd PCT/PTO 08 DEC 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of Little *et al.*
☐ Patent of:

☒ Application No.: To be assigned
☐ Patent No.:

☐ Filed:
☐ Issued:

For: MULTIVALENT ANTIBODY CONSTRUCTS

Group Art Unit: To be assigned

Examiner: To be assigned

Attorney Docket No.: 8484-089-999

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(d)] - Nonprofit Organization

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of organization Deutsches Krebsforschungszentrum Stiftung des öffentlichen Rechts
Address of organization Im Neuenheimer Feld 280, D-69120 Heidelberg Germany

Type of organization
- ☐ University or other institution of higher education
- ☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
- ☐ Nonprofit scientific or educational under statute of state of the United States of America
  (Name of state ______)
  (Citation of statute ______)
- ☒ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) if located in the United States of America.
- ☐ Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
  (Name of state ______)
  (Citation of statute ______)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **MULTIVALENT ANTIBODY CONSTRUCTS** by inventor(s) Melvyn Little and Sergej Kipriyanov described in

☐ the specification filed herewith
☐ application no. filed
☐ patent no. issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above and/or there is an obligation under contract or law by the inventor(s) to convey rights to the nonprofit organization identified above with regard to the invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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FULL NAME____________________
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

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Name of person signing Prof. Dr. Harald zur Hausen Dr. Jos[illegible]
Title of person other than owner Scientific Member of the [illegible]ber of the
Address of person signing Management Board [illegible]ment Board

Signature____________________ Date 21. Nov 00

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

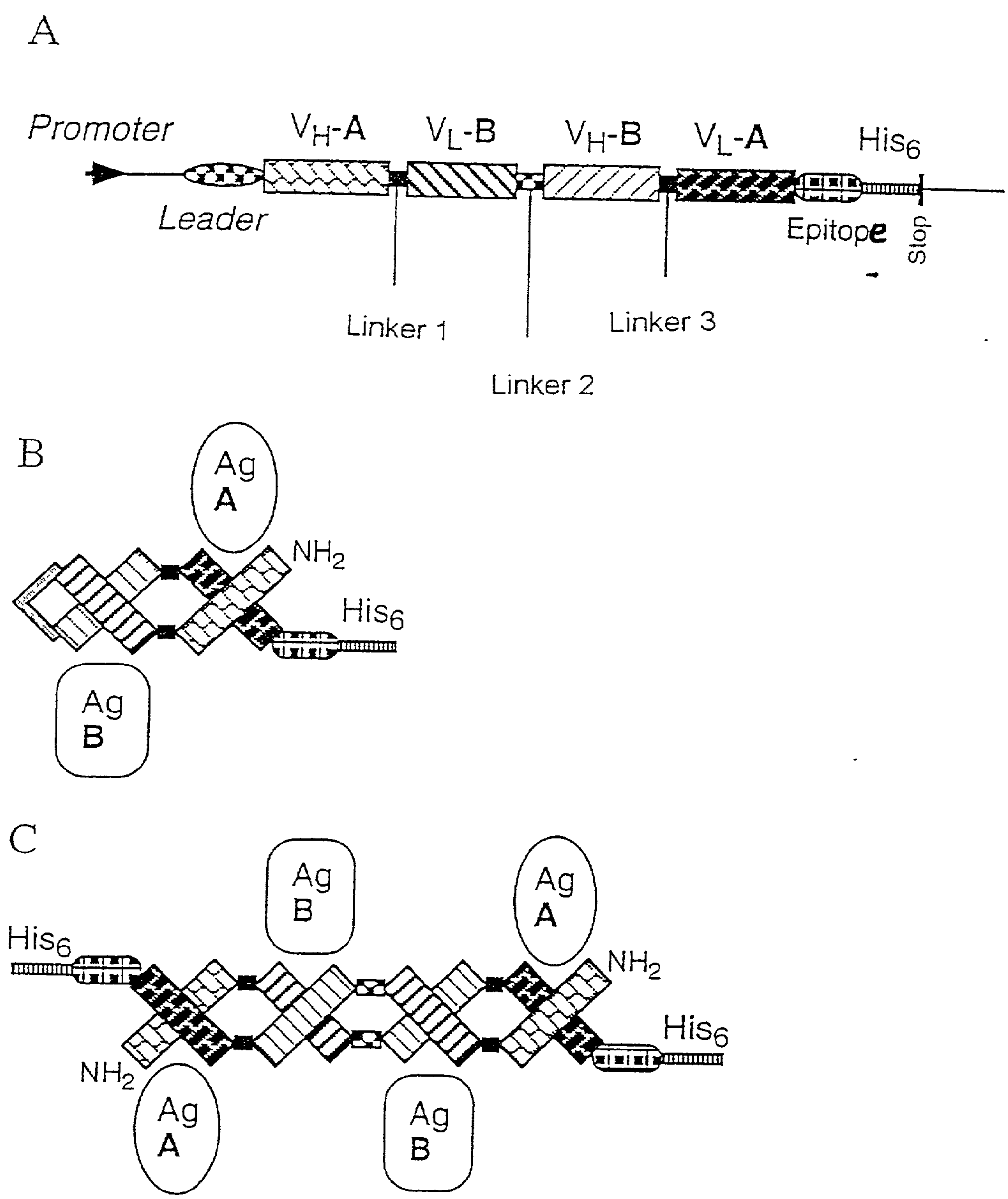
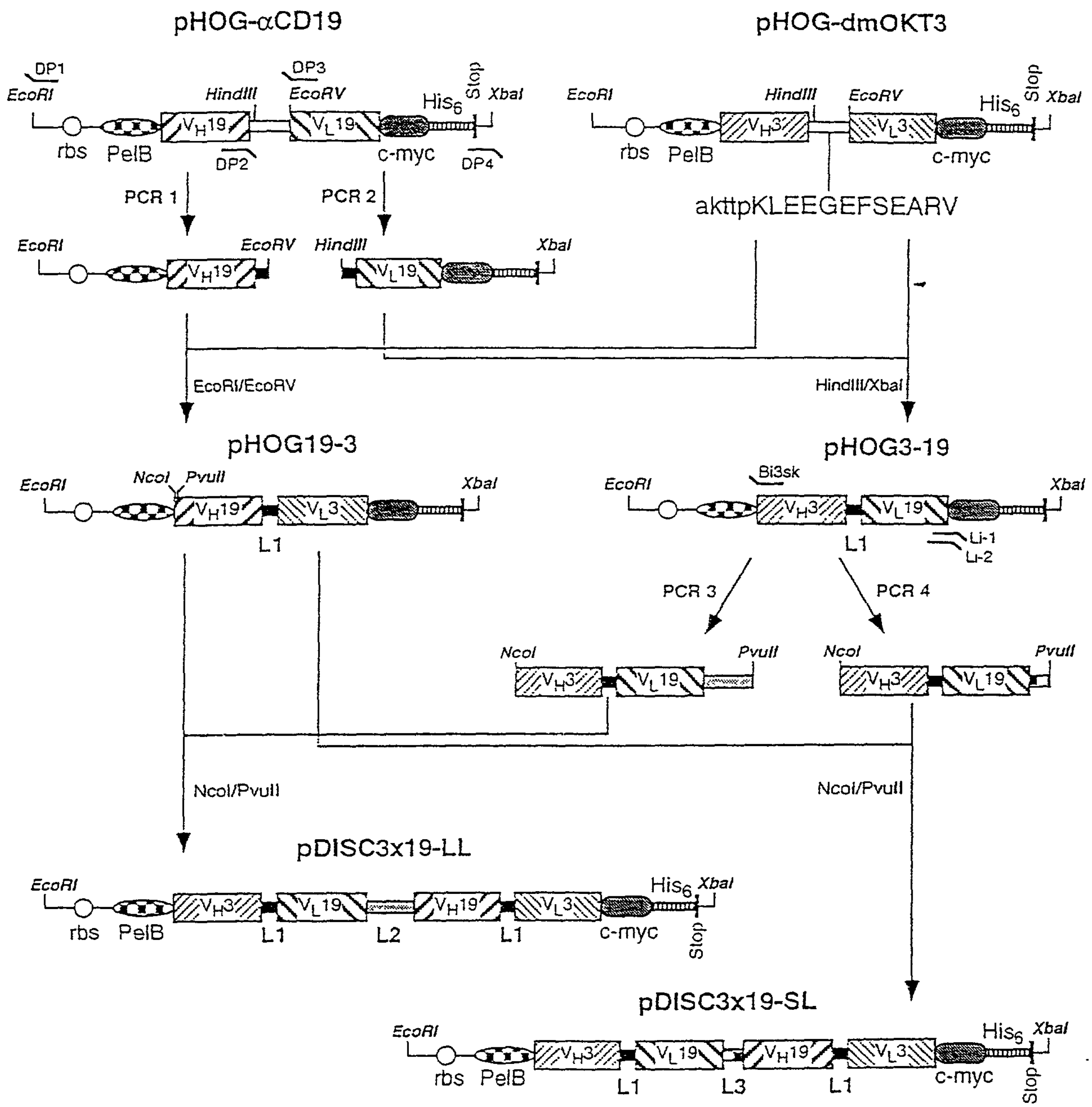

A
Promoter
Leader
V_H-A
V_L-B
V_H-B
V_L-A
His_6
Epitope
Stop
Linker 1
Linker 2
Linker 3
B
Ag A
NH_2
His_6
Ag B
C
Ag B
Ag A
His_6
NH_2
His_6
NH_2
Ag A
Ag B

FIGURE 1



Linkers: L1 = GG
L2 = $(G_4S)_4$
L3 = GGPGS

FIGURE 2

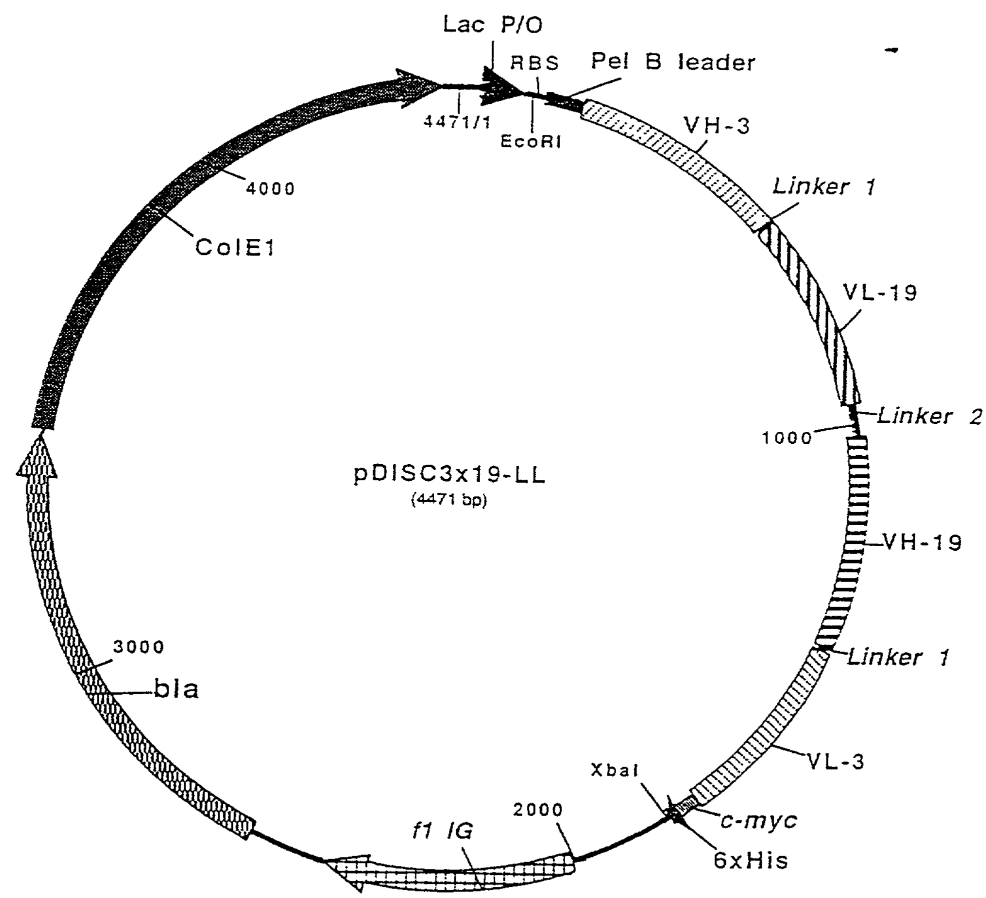

Lac P/O
RBS
Pel B leader
4471/1
EcoRI
VH-3
Linker 1
4000
ColE1
VL-19
Linker 2
1000
pDISC3x19-LL
(4471 bp)
VH-19
3000
bla
Linker 1
VL-3
XbaI
2000
f1 IG
c-myc
6xHis

FIGURE 3

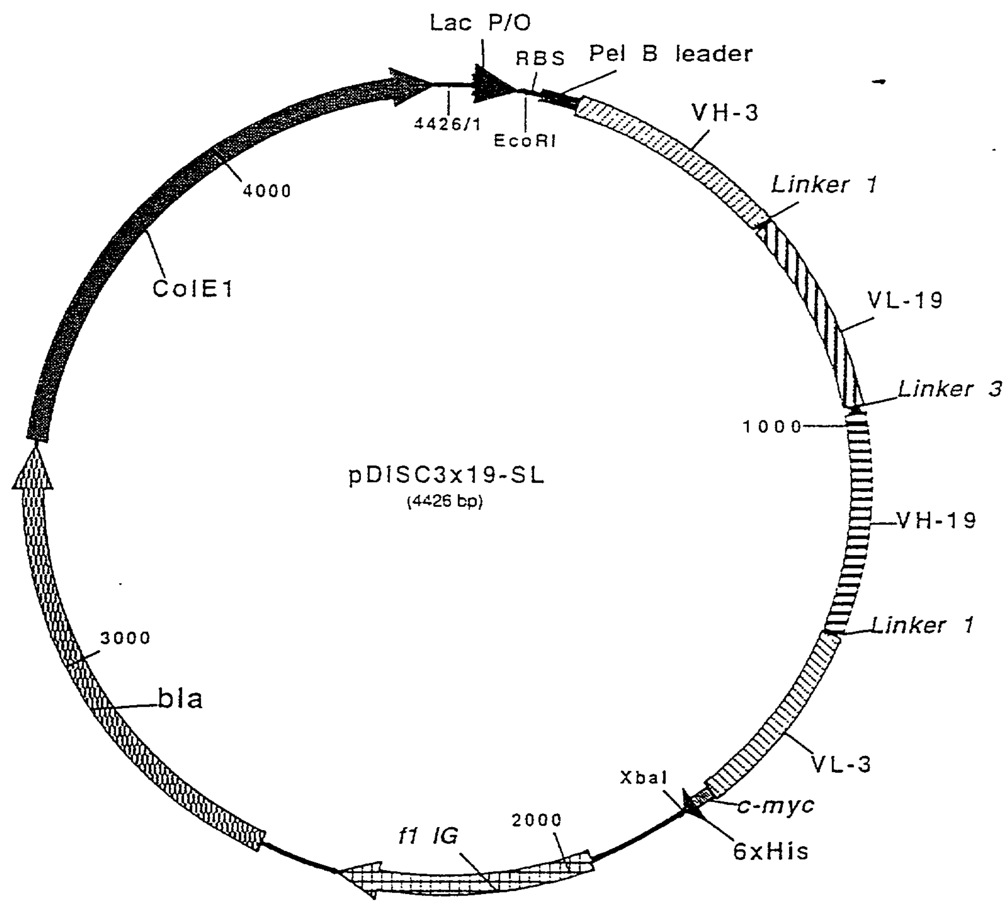

Lac P/O
RBS
Pel B leader
VH-3
4426/1
EcoRI
Linker 1
4000
VL-19
ColE1
Linker 3
1000
pDISC3x19-SL
(4426 bp)
VH-19
Linker 1
3000
bla
VL-3
XbaI
c-myc
f1 IG
2000
6xHis

FIGURE 4

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```
EcoRI       RBS              PelB leader                                        NcoI
1    GAATTCATTAAAGAGGAGAAATTAACCATGAAATACCTATTGCCTACGGCAGCCGCTGGCTTGCTGCTGCTGGCAGCTCAGCCGGCCATGG
                             1▸ M  K  Y  L  L  P  T  A  A  A  G  L  L  L  L  A  A  Q  P  A  M
      +               Frame-H1                                  VH  anti-CD3
92   CGCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTAC
22▸  A  Q  V  Q  L  Q  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T
        CDR-H1          Frame-H2                                CDR-H2
183  TAGGTACACGATGCACTGGGTAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATAC
52▸   R  Y  T  M  H  W  V  K  Q  R  P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  R  G  Y  T
                                  Frame-H3
267  TAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGACTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGAC
30▸   N  Y  N  Q  K  F  K  D  K  A  T  L  T  T  D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T
                                            CDR-H3                          Frame-H4
354  ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGATATTATGATGATCATTACAGCCTTGACTACTGGGGCCAAGGCACCACTCTCA
109▸  S  E  D  S  A  V  Y  Y  C  A  R  Y  Y  D  D  H  Y  S  L  D  Y  W  G  Q  G  T  T  L
               CH1              Linker 1       Frame-L1                        VL  anti-CD19
440  CAGTCTCCTCAGCCAAAACAACACCCAAGCTTGGCGGTGATATCTTGCTCACCCAAACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGA
138▸ T  V  S  S  A  K  T  T  P  K  L  G  G  D  I  L  L  T  Q  T  P  A  S  L  A  V  S  L  G  Q
                    CDR-L1                                                    Frame-L2
530  GGGCCACCATCTCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACAGATTCCAGGAC
168▸ R  A  T  I  S  C  K  A  S  Q  S  V  D  Y  D  G  D  S  Y  L  N  W  Y  Q  Q  I  P  G
                        CDR-L2                           Frame-L3
614  AGCCACCCAAACTCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTT
196▸ Q  P  P  K  L  L  I  Y  D  A  S  N  L  V  S  G  I  P  P  R  F  S  G  S  G  S  G  T  D  F
                                                       CDR-L3                     Frame-L4
702  CACCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCTATCACTGTCAGCAAAGTACTGAGGATCCGTGGACGTTCGGTGGA
225▸  T  L  N  I  H  P  V  E  K  V  D  A  A  T  Y  H  C  Q  Q  S  T  E  D  P  W  T  F  G  G
                    C kacca      NotI                        Linker 2
790  GGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCGGCCGCTGGTGGTGGTGGTTCTGGCGGCGGTGGTAGCGGTGGTGGCGGC
255▸ G  T  K  L  E  I  K  R  A  D  A  A  A  A  G  G  G  G  S  G  G  G  G  S  G  G  G  G
                              PvuII      Frame-H1          VH  anti-CD19
874  TCCGGTGGTGGTGGTAGCCAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCCTCAGTGAAGATTTCCTGCAAGG
283▸ S  G  G  G  G  S  Q  V  Q  L  Q  Q  S  G  A  E  L  V  R  P  G  S  S  V  K  I  S  C  K
                    CDR-H1              Frame-H2                                      CDR-H2
962  CTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGCCTGGACAGGGTCTTGAGTGGATTGGACAGATTTGGC
312▸ A  S  G  Y  A  F  S  S  Y  W  M  N  W  V  K  Q  R  P  G  Q  G  L  E  W  I  G  Q  I  W
                                                          PstI      Frame-H3
1049 CTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGTAAAGCCACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACA
341▸ P  G  D  G  D  T  N  Y  N  G  K  F  K  G  K  A  T  L  T  A  D  E  S  S  S  T  A  Y
                                                          CDR-H3
1133 TGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGACGGGAGACTACGACGGTAGGCCGTTATTACTAT
369▸ M  Q  L  S  S  L  A  S  E  D  S  A  V  Y  F  C  A  R  R  E  T  T  T  V  G  R  Y  Y  Y
                 Frame-H4                      CH1                Linker 1      Frame-L1
1219 GCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACAACACCCAAGCTTGGCGGTGATATCGTGCTCACTC
398▸  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S  A  K  T  T  P  K  L  G  G  D  I  V  L  T
  VL  anti-CD3                                               CDR-L1
1307 AGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGG
427▸ Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  M  T  C  S  A  S  S  S  V  S  Y  M  N  W
  Frame-L2                                       CDR-L2                   Frame-L3
1393 TACCAGCAGAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCACTTCAGGGGCA
456▸  Y  Q  Q  K  S  G  T  S  P  K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A  H  F  R  G
                                                                           CDR-L3
1481 GTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAA
485▸ S  G  S  G  T  S  Y  S  L  T  I  S  G  M  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  S  N
                  Frame-L4                    C kappa                      c-myc  epitope
1569 CCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGCACCAACTGGATCCGAACAAAAGCTGATCTCAG
514▸  P  F  T  F  G  S  G  T  K  L  E  I  N  R  A  D  T  A  P  T  G  S  E  Q  K  L  I  S
                        His6 tail         XbaI
1655 AAGAAGACCTAAACTCACATCACCATCACCATCACTAATCTAGA
543▸ E  E  D  L  N  S  H  H  H  H  H  H  •
```

FIGURE 5

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```
     EcoRI       RBS              PelB leader                                           NcoI
   1 GAATTCATTAAAGAGGAGAAATTAACCATGAAATACCTATTGCCTACGGCAGCCGCTGGCTTGCTGCTGCTGGCAGCTCAGCCGGCCATGG
                                 1> M  K  Y  L  L  P  T  A  A  A  G  L  L  L  L  A  A  Q  P  A  M
        +                    Frame-H1                                          VH  anti-CD3
  92 CGCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTAC
 22> A  Q  V  Q  L  Q  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T
         CDR-H1             Frame-H2                                     CDR-H2
 183 TAGGTACACGATGCACTGGGTAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATAC
 52>   R  Y  T  M  H  W  V  K  Q  R  P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  R  G  Y  T
                                       Frame-H3
 267 TAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGACTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGAC
 80>   N  Y  N  Q  K  F  K  D  K  A  T  L  T  T  D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T
                                                CDR-H3                         Frame-H4
 354 ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGATATTATGATGATCATTACAGCCTTGACTACTGGGGCCAAGGCACCACTCTCA
109>   S  E  D  S  A  V  Y  Y  C  A  R  Y  Y  D  D  H  Y  S  L  D  Y  W  G  Q  G  T  T  L
               CH1                 Linker 1       Frame-L1                    VL  anti-CD19
 440 CAGTCTCCTCAGCCAAAACAACACCCAAGCTTGGCGGTGATATCTTGCTCACCCAAACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGA
138> T  V  S  S  A  K  T  T  P  K  L  G  G  D  I  L  L  T  Q  T  P  A  S  L  A  V  S  L  G  Q
                       CDR-L1                                                   Frame-L2
 530 GGGCCACCATCTCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACAGATTCCAGGAC
168> R  A  T  I  S  C  K  A  S  Q  S  V  D  Y  D  G  D  S  Y  L  N  W  Y  Q  Q  I  P  G
                             CDR-L2                        Frame-L3
 614 AGCCACCCAAACTCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTT
196> Q  P  P  K  L  L  I  Y  D  A  S  N  L  V  S  G  I  P  P  R  F  S  G  S  G  S  G  T  D  F
                                                             CDR-L3               Frame-L4
 702 CACCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCTATCACTGTCAGCAAAGTACTGAGGATCCGTGGACGTTCGGTGGA
225>   T  L  N  I  H  P  V  E  K  V  D  A  A  T  Y  H  C  Q  Q  S  T  E  D  P  W  T  F  G  G
                        C kappa       NotI         Linker 3              PvuII   Frame-H1
 790 GGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCGGCCGCTGGTGGCCCAGGGTCGCAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCT
255>   G  T  K  L  E  I  K  R  A  D  A  A  A  A  G  G  P  G  S  Q  V  Q  L  Q  Q  S  G  A  E  L
     VH  anti-CD19                                               CDR-H1              Frame-H2
 879 GGTGAGGCCTGGGTCCTCAGTGAAGATTTCCTGCAAGGCTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGC
284>   V  R  P  G  S  S  V  K  I  S  C  K  A  S  G  Y  A  F  S  S  Y  W  M  N  W  V  K  Q  R
                                 CDR-H2
 968 CTGGACAGGGTCTTGAGTGGATTGGACAGATTTGGCCTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGTAAAGCC
314> P  G  Q  G  L  E  W  I  G  Q  I  W  P  G  D  G  D  T  N  Y  N  G  K  F  K  G  K  A
             Frame-H3
1051 ACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGAC
342>   T  L  T  A  D  E  S  S  S  T  A  Y  M  Q  L  S  S  L  A  S  E  D  S  A  V  Y  F  C  A  R
        CDR-H3                                              Frame-H4                        CH1
1142 GGGAGACTACGACGGTAGGCCGTTATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAA
372> R  E  T  T  T  V  G  R  Y  Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S  A  K
              Linker 1       Frame-L1       VL  anti-CD3
1226 CAACACCCAAGCTTGGCGGTGATATCGTGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCA
400> T  T  P  K  L  G  G  D  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  M  T  C
        CDR-L1                        Frame-L2                                            CDR-L2
1316 GTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCAGAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGACACATCCAA
430> S  A  S  S  S  V  S  Y  M  N  W  Y  Q  Q  K  S  G  T  S  P  K  R  W  I  Y  D  T  S  K
                Frame-L3
1401 ACTGGCTTCTGGAGTCCCTGCTCACTTCAGGGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGC
458>   L  A  S  G  V  P  A  H  F  R  G  S  G  S  G  T  S  Y  S  L  T  I  S  G  M  E  A  E  D  A
                 CDR-L3                            Frame-L4                        C kappa
1491 TGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGC
488>   A  T  Y  Y  C  Q  Q  W  S  S  N  P  F  T  F  G  S  G  T  K  L  E  I  N  R  A  D  T  A
                 c-myc  epitope                             His6 tail        XbaI
1578 ACCAACTGGATCCGAACAAAAGCTGATCTCAGAAGAAGACCTAAACTCACATCACCATCACCATCACTAATCTAGA
517>   P  T  G  S  E  Q  K  L  I  S  E  E  D  L  N  S  H  H  H  H  H  H  ·
```

FIGURE 6

941 ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTAC
1▸ M R F P S I F T A V L F A A S S A L A A P V N T T

alpha-factor signal

1015 AACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATG
25▸ T E D E T A Q I P A E A V I G Y S D L E G D F D

1089 TTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
50▸ V A V L P F S N S T N N G L L F I N T T I A S I A

XhoI EcoRI

1163 GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCCAGGTGCAACTGCAGCAGTC
75▸ A K E E G V S L E K R E A E A E F Q V Q L Q Q S

VH anti-CD3

1234 TGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
98▸ G A E L A R P G A S V K M S C K A S

FIGURE 7

```
941  ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTAC
  1▸ M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T

                                  alpha-factor signal
1015 AACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATG
 25▸  T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D

                                                                          BsrDI
1089 TTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
 50▸ V  A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A

                                                    EcoRI
                         XhoI
1163 GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCATGGCGCAGGTGCAACTGCAG
 75▸  A  K  E  E  G  V  S  L  E  K  R  E  A  E  A  E  F  M  A  Q  V  Q  L  Q

         VH  anti-CD3
1235 CAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCT
 99▸  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  M  S  C  K  A  S
```

FIGURE 8

09/ 674794

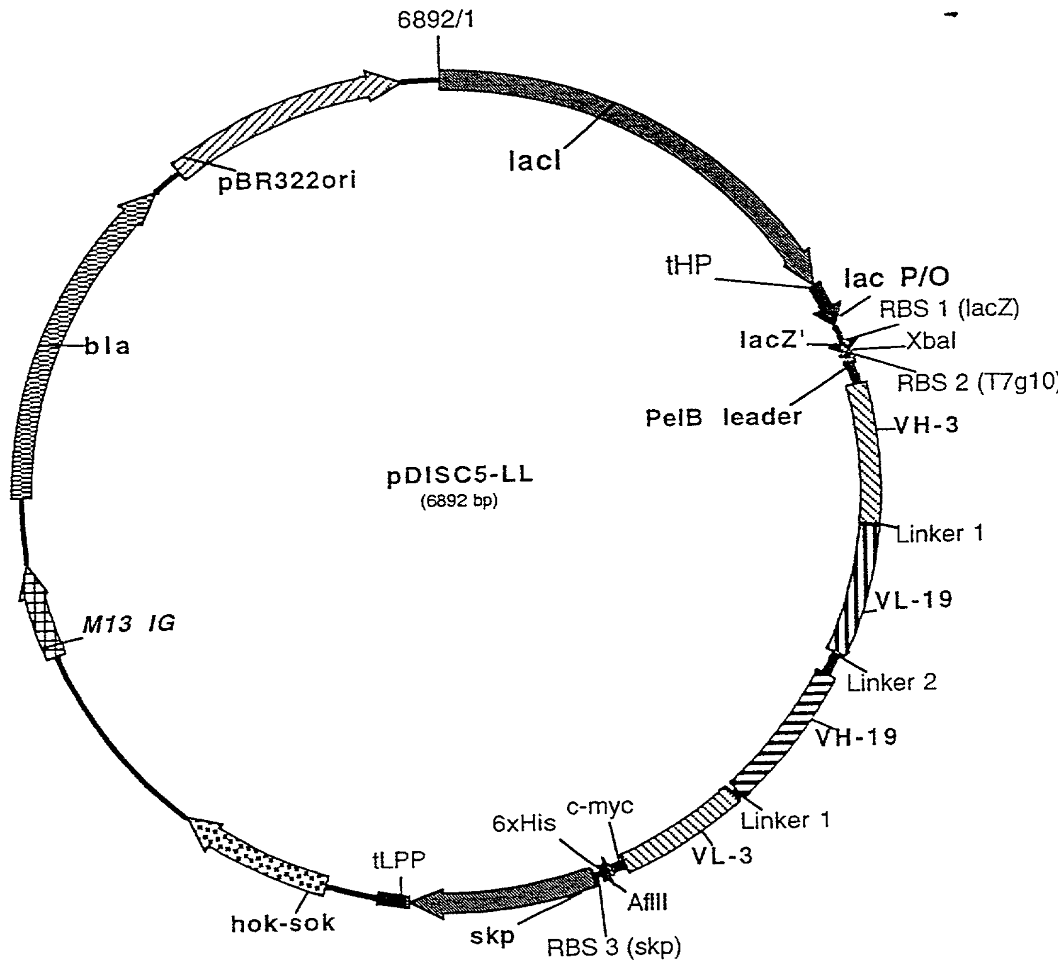

FIGURE 9

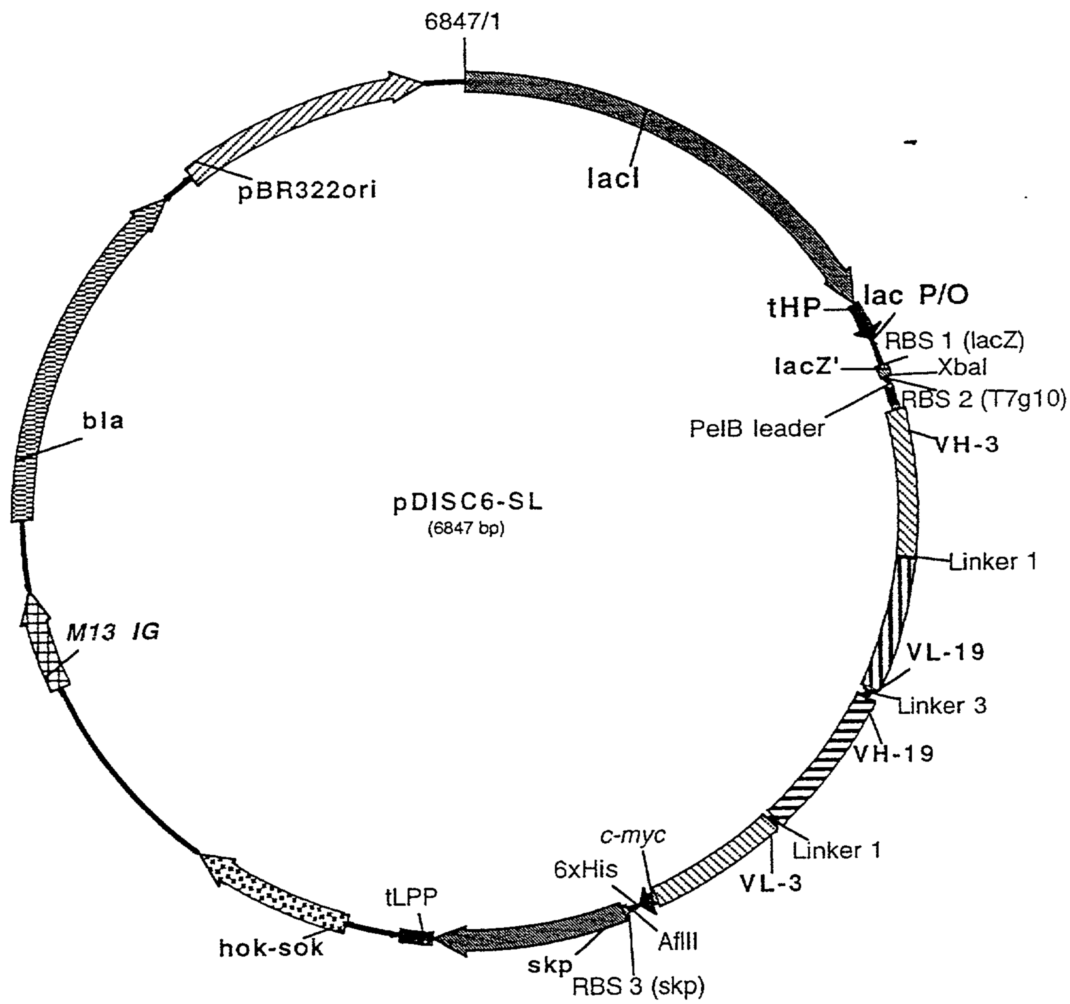
6847/1
lacI
tHP
lac P/O
RBS 1 (lacZ)
lacZ'
XbaI
RBS 2 (T7g10)
PelB leader
VH-3
pDISC6-SL
(6847 bp)
Linker 1
VL-19
Linker 3
VH-19
Linker 1
VL-3
c-myc
6xHis
AflII
skp
RBS 3 (skp)
tLPP
hok-sok
M13 IG
bla
pBR322ori

FIGURE 10

SEQUENCE RECORD

(1) GENERAL INDICATIONS:

(i) APPLICANT:

(A) NAME: Deutsches Krebsforschungszentrum
(B) STREET: Im Neuenheimer Feld 280
(C) TOWN: Heidelberg
(E) COUNTRY: Germany
(F) POSTAL CODE: 69120

(ii) TITLE OF THE INVENTION: Multivalent Antibody Constructs

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER-READABLE VERSION:

(A) DATA CARRIER: floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, version #1.30 (EPA)

(2) INDICATIONS AS TO SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1698 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear

(ii) KIND OF MOLECULE: genome DNA

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) POSITION: 28..1689

(ix) FEATURE:

(A) NAME/KEY: mat_peptide
(B) POSITION: 28..1689

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GAATTCATTA AAGAGGAGAA ATTAACC ATG AAA TAC CTA TTG CCT ACG GCA     51
                              Met Lys Tyr Leu Leu Pro Thr Ala
                               1               5
```

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GCC GCT GGC TTG CTG CTG CTG GCA GCT CAG CCG GCC ATG GCG CAG GTG       99
Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala Gln Val
     10                  15                  20

CAA CTG CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG      147
Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val
 25                  30                  35                  40

AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTT ACT AGG TAC ACG ATG      195
Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr Thr Met
                 45                  50                  55

CAC TGG GTA AAA CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT GGA TAC      243
His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Tyr
             60                  65                  70

ATT AAT CCT AGC CGT GGT TAT ACT AAT TAC AAT CAG AAG TTC AAG GAC      291
Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe Lys Asp
         75                  80                  85

AAG GCC ACA TTG ACT ACA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAA      339
Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln
     90                  95                 100

CTG AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA      387
Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg
105                 110                 115                 120

TAT TAT GAT GAT CAT TAC AGC CTT GAC TAC TGG GGC CAA GGC ACC ACT      435
Tyr Tyr Asp Asp His Tyr Ser Leu Asp Tyr Trp Gly Gln Gly Thr Thr
                125                 130                 135

CTC ACA GTC TCC TCA GCC AAA ACA ACA CCC AAG CTT GGC GGT GAT ATC      483
Leu Thr Val Ser Ser Ala Lys Thr Thr Pro Lys Leu Gly Gly Asp Ile
            140                 145                 150

TTG CTC ACC CAA ACT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG      531
Leu Leu Thr Gln Thr Pro Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
        155                 160                 165

GCC ACC ATC TCC TGC AAG GCC AGC CAA AGT GTT GAT TAT GAT GGT GAT      579
Ala Thr Ile Ser Cys Lys Ala Ser Gln Ser Val Asp Tyr Asp Gly Asp
    170                 175                 180

AGT TAT TTG AAC TGG TAC CAA CAG ATT CCA GGA CAG CCA CCC AAA CTC      627
Ser Tyr Leu Asn Trp Tyr Gln Gln Ile Pro Gly Gln Pro Pro Lys Leu
185                 190                 195                 200

CTC ATC TAT GAT GCA TCC AAT CTA GTT TCT GGG ATC CCA CCC AGG TTT      675
Leu Ile Tyr Asp Ala Ser Asn Leu Val Ser Gly Ile Pro Pro Arg Phe
                205                 210                 215

AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG      723
Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His Pro Val
            220                 225                 230
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GAG AAG GTG GAT GCT GCA ACC TAT CAC TGT CAG CAA AGT ACT GAG GAT          771
Glu Lys Val Asp Ala Ala Thr Tyr His Cys Gln Gln Ser Thr Glu Asp
        235                 240                 245

CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT GAT          819
Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp
    250                 255                 260

GCT GCG GCC GCT GGT GGT GGT GGT TCT GGC GGC GGT GGT AGC GGT GGT          867
Ala Ala Ala Ala Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
265                 270                 275                 280

GGC GGC TCC GGT GGT GGT GGT AGC CAG GTG CAG CTG CAG CAG TCT GGG          915
Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln Leu Gln Gln Ser Gly
                285                 290                 295

GCT GAG CTG GTG AGG CCT GGG TCC TCA GTG AAG ATT TCC TGC AAG GCT          963
Ala Glu Leu Val Arg Pro Gly Ser Ser Val Lys Ile Ser Cys Lys Ala
            300                 305                 310

TCT GGC TAT GCA TTC AGT AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG         1011
Ser Gly Tyr Ala Phe Ser Ser Tyr Trp Met Asn Trp Val Lys Gln Arg
        315                 320                 325

CCT GGA CAG GGT CTT GAG TGG ATT GGA CAG ATT TGG CCT GGA GAT GGT         1059
Pro Gly Gln Gly Leu Glu Trp Ile Gly Gln Ile Trp Pro Gly Asp Gly
    330                 335                 340

GAT ACT AAC TAC AAT GGA AAG TTC AAG GGT AAA GCC ACT CTG ACT GCA         1107
Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala
345                 350                 355                 360

GAC GAA TCC TCC AGC ACA GCC TAC ATG CAA CTC AGC AGC CTA GCA TCT         1155
Asp Glu Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Ala Ser
                365                 370                 375

GAG GAC TCT GCG GTC TAT TTC TGT GCA AGA CGG GAG ACT ACG ACG GTA         1203
Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Arg Glu Thr Thr Thr Val
            380                 385                 390

GGC CGT TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC         1251
Gly Arg Tyr Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
        395                 400                 405

ACC GTC TCC TCA GCC AAA ACA ACA CCC AAG CTT GGC GGT GAT ATC GTG         1299
Thr Val Ser Ser Ala Lys Thr Thr Pro Lys Leu Gly Gly Asp Ile Val
    410                 415                 420

CTC ACT CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AAG GTC         1347
Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys Val
425                 430                 435                 440

ACC ATG ACC TGC AGT GCC AGC TCA AGT GTA AGT TAC ATG AAC TGG TAC         1395
Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met Asn Trp Tyr
                445                 450                 455
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CAG CAG AAG TCA GGC ACC TCC CCC AAA AGA TGG ATT TAT GAC ACA TCC 1443
Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr Asp Thr Ser
460 465 470

AAA CTG GCT TCT GGA GTC CCT GCT CAC TTC AGG GGC AGT GGG TCT GGG 1491
Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser Gly Ser Gly
475 480 485

ACC TCT TAC TCT CTC ACA ATC AGC GGC ATG GAG GCT GAA GAT GCT GCC 1539
Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu Asp Ala Ala
490 495 500

ACT TAT TAC TGC CAG CAG TGG AGT AGT AAC CCA TTC ACG TTC GGC TCG 1587
Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr Phe Gly Ser
505 510 515 520

GGG ACA AAG TTG GAA ATA AAC CGG GCT GAT ACT GCA CCA ACT GGA TCC 1635
Gly Thr Lys Leu Glu Ile Asn Arg Ala Asp Thr Ala Pro Thr Gly Ser
525 530 535

GAA CAA AAG CTG ATC TCA GAA GAA GAC CTA AAC TCA CAT CAC CAT CAC 1683
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser His His His His
540 545 550

CAT CAC TAATCTAGA 1698
His His

(2) INDICATIONS AS TO ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 554 amino acids
(B) KIND: amino acid
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala
1 5 10 15

Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln Ser Gly Ala Glu
20 25 30

Leu Ala Arg Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly
35 40 45

Tyr Thr Phe Thr Arg Tyr Thr Met His Trp Val Lys Gln Arg Pro Gly
50 55 60

Gln Gly Leu Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr
65 70 75 80

```
Asn Tyr Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys
                85                  90                  95

Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp
            100                 105                 110

Ser Ala Val Tyr Tyr Cys Ala Arg Tyr Tyr Asp Asp His Tyr Ser Leu
        115                 120                 125

Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr
    130                 135                 140

Thr Pro Lys Leu Gly Gly Asp Ile Leu Leu Thr Gln Thr Pro Ala Ser
145                 150                 155                 160

Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Lys Ala Ser
                165                 170                 175

Gln Ser Val Asp Tyr Asp Gly Asp Ser Tyr Leu Asn Trp Tyr Gln Gln
            180                 185                 190

Ile Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Asp Ala Ser Asn Leu
        195                 200                 205

Val Ser Gly Ile Pro Pro Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
    210                 215                 220

Phe Thr Leu Asn Ile His Pro Val Glu Lys Val Asp Ala Ala Thr Tyr
225                 230                 235                 240

His Cys Gln Gln Ser Thr Glu Asp Pro Trp Thr Phe Gly Gly Gly Thr
                245                 250                 255

Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala Ala Gly Gly Gly Gly
            260                 265                 270

Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
        275                 280                 285

Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Ser
    290                 295                 300

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Tyr
305                 310                 315                 320

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
                325                 330                 335

Gly Gln Ile Trp Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe
            340                 345                 350

Lys Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser Thr Ala Tyr
        355                 360                 365
```

Met Gln Leu Ser Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Phe Cys
370 375 380

Ala Arg Arg Glu Thr Thr Thr Val Gly Arg Tyr Tyr Tyr Ala Met Asp
385 390 395 400

Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr
405 410 415

Pro Lys Leu Gly Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ile Met
420 425 430

Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser
435 440 445

Ser Val Ser Tyr Met Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser Pro
450 455 460

Lys Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ala
465 470 475 480

His Phe Arg Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser
485 490 495

Gly Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser
500 505 510

Ser Asn Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn Arg
515 520 525

Ala Asp Thr Ala Pro Thr Gly Ser Glu Gln Lys Leu Ile Ser Glu Glu
530 535 540

Asp Leu Asn Ser His His His His His His
545 550

(2) INDICATIONS AS TO ID NO: 3:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1653 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: genome DNA
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) POSITION: 28..1644

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) POSITION: 28..1644

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GAATTCATTA AAGAGGAGAA ATTAACC ATG AAA TAC CTA TTG CCT ACG GCA         51
                              Met Lys Tyr Leu Leu Pro Thr Ala
                                1               5

GCC GCT GGC TTG CTG CTG CTG GCA GCT CAG CCG GCC ATG GCG CAG GTG        99
Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala Gln Val
     10                  15                  20

CAA CTG CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG       147
Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val
 25                  30                  35                  40

AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTT ACT AGG TAC ACG ATG       195
Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr Thr Met
                 45                  50                  55

CAC TGG GTA AAA CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT GGA TAC       243
His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Tyr
             60                  65                  70

ATT AAT CCT AGC CGT GGT TAT ACT AAT TAC AAT CAG AAG TTC AAG GAC       291
Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe Lys Asp
         75                  80                  85

AAG GCC ACA TTG ACT ACA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAA       339
Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln
     90                  95                 100

CTG AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA       387
Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg
105                 110                 115                 120

TAT TAT GAT GAT CAT TAC AGC CTT GAC TAC TGG GGC CAA GGC ACC ACT       435
Tyr Tyr Asp Asp His Tyr Ser Leu Asp Tyr Trp Gly Gln Gly Thr Thr
                125                 130                 135

CTC ACA GTC TCC TCA GCC AAA ACA ACA CCC AAG CTT GGC GGT GAT ATC       483
Leu Thr Val Ser Ser Ala Lys Thr Thr Pro Lys Leu Gly Gly Asp Ile
            140                 145                 150

TTG CTC ACC CAA ACT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG       531
Leu Leu Thr Gln Thr Pro Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
        155                 160                 165

GCC ACC ATC TCC TGC AAG GCC AGC CAA AGT GTT GAT TAT GAT GGT GAT       579
Ala Thr Ile Ser Cys Lys Ala Ser Gln Ser Val Asp Tyr Asp Gly Asp
    170                 175                 180
```

AGT TAT TTG AAC TGG TAC CAA CAG ATT CCA GGA CAG CCA CCC AAA CTC 627
Ser Tyr Leu Asn Trp Tyr Gln Gln Ile Pro Gly Gln Pro Pro Lys Leu
185 190 195 200

CTC ATC TAT GAT GCA TCC AAT CTA GTT TCT GGG ATC CCA CCC AGG TTT 675
Leu Ile Tyr Asp Ala Ser Asn Leu Val Ser Gly Ile Pro Pro Arg Phe
205 210 215

AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG 723
Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His Pro Val
220 225 230

GAG AAG GTG GAT GCT GCA ACC TAT CAC TGT CAG CAA AGT ACT GAG GAT 771
Glu Lys Val Asp Ala Ala Thr Tyr His Cys Gln Gln Ser Thr Glu Asp
235 240 245

CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT GAT 819
Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp
250 255 260

GCT GCG GCC GCT GGT GGC CCA GGG TCG CAG GTG CAG CTG CAG CAG TCT 867
Ala Ala Ala Ala Gly Gly Pro Gly Ser Gln Val Gln Leu Gln Gln Ser
265 270 275 280

GGG GCT GAG CTG GTG AGG CCT GGG TCC TCA GTG AAG ATT TCC TGC AAG 915
Gly Ala Glu Leu Val Arg Pro Gly Ser Ser Val Lys Ile Ser Cys Lys
285 290 295

GCT TCT GGC TAT GCA TTC AGT AGC TAC TGG ATG AAC TGG GTG AAG CAG 963
Ala Ser Gly Tyr Ala Phe Ser Ser Tyr Trp Met Asn Trp Val Lys Gln
300 305 310

AGG CCT GGA CAG GGT CTT GAG TGG ATT GGA CAG ATT TGG CCT GGA GAT 1011
Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Gln Ile Trp Pro Gly Asp
315 320 325

GGT GAT ACT AAC TAC AAT GGA AAG TTC AAG GGT AAA GCC ACT CTG ACT 1059
Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu Thr
330 335 340

GCA GAC GAA TCC TCC AGC ACA GCC TAC ATG CAA CTC AGC AGC CTA GCA 1107
Ala Asp Glu Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Ala
345 350 355 360

TCT GAG GAC TCT GCG GTC TAT TTC TGT GCA AGA CGG GAG ACT ACG ACG 1155
Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Arg Glu Thr Thr Thr
365 370 375

GTA GGC CGT TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA 1203
Val Gly Arg Tyr Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser
380 385 390

GTC ACC GTC TCC TCA GCC AAA ACA ACA CCC AAG CTT GGC GGT GAT ATC 1251
Val Thr Val Ser Ser Ala Lys Thr Thr Pro Lys Leu Gly Gly Asp Ile
395 400 405

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GTG CTC ACT CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AAG        1299
Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Lys
    410                 415                     420

GTC ACC ATG ACC TGC AGT GCC AGC TCA AGT GTA AGT TAC ATG AAC TGG        1347
Val Thr Met Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met Asn Trp
425                 430                     435                 440

TAC CAG CAG AAG TCA GGC ACC TCC CCC AAA AGA TGG ATT TAT GAC ACA        1395
Tyr Gln Gln Lys Ser Gly Thr Ser Pro Lys Arg Trp Ile Tyr Asp Thr
                445                     450                 455

TCC AAA CTG GCT TCT GGA GTC CCT GCT CAC TTC AGG GGC AGT GGG TCT        1443
Ser Lys Leu Ala Ser Gly Val Pro Ala His Phe Arg Gly Ser Gly Ser
            460                     465                 470

GGG ACC TCT TAC TCT CTC ACA ATC AGC GGC ATG GAG GCT GAA GAT GCT        1491
Gly Thr Ser Tyr Ser Leu Thr Ile Ser Gly Met Glu Ala Glu Asp Ala
        475                 480                     485

GCC ACT TAT TAC TGC CAG CAG TGG AGT AGT AAC CCA TTC ACG TTC GGC        1539
Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr Phe Gly
    490                 495                     500

TCG GGG ACA AAG TTG GAA ATA AAC CGG GCT GAT ACT GCA CCA ACT GGA        1587
Ser Gly Thr Lys Leu Glu Ile Asn Arg Ala Asp Thr Ala Pro Thr Gly
505                     510                     515                 520

TCC GAA CAA AAG CTG ATC TCA GAA GAA GAC CTA AAC TCA CAT CAC CAT        1635
Ser Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser His His His
                525                     530                     535

CAC CAT CAC TAATCTAGA                                                  1653
His His His
```

(2) INDICATIONS AS TO ID NO: 4:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 539 amino acids
  - (B) KIND: amino acid
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala
 1               5                  10                  15

Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln Ser Gly Ala Glu
            20                  25                  30

Leu Ala Arg Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly
        35                  40                  45
```

Tyr Thr Phe Thr Arg Tyr Thr Met His Trp Val Lys Gln Arg Pro Gly
50 55 60

Gln Gly Leu Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr
65 70 75 80

Asn Tyr Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys
85 90 95

Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp
100 105 110

Ser Ala Val Tyr Tyr Cys Ala Arg Tyr Tyr Asp Asp His Tyr Ser Leu
115 120 125

Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr
130 135 140

Thr Pro Lys Leu Gly Gly Asp Ile Leu Leu Thr Gln Thr Pro Ala Ser
145 150 155 160

Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Lys Ala Ser
165 170 175

Gln Ser Val Asp Tyr Asp Gly Asp Ser Tyr Leu Asn Trp Tyr Gln Gln
180 185 190

Ile Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Asp Ala Ser Asn Leu
195 200 205

Val Ser Gly Ile Pro Pro Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
210 215 220

Phe Thr Leu Asn Ile His Pro Val Glu Lys Val Asp Ala Ala Thr Tyr
225 230 235 240

His Cys Gln Gln Ser Thr Glu Asp Pro Trp Thr Phe Gly Gly Gly Thr
245 250 255

Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala Ala Ala Gly Gly Pro Gly
260 265 270

Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly
275 280 285

Ser Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser
290 295 300

Tyr Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp
305 310 315 320

Ile Gly Gln Ile Trp Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys
325 330 335

```
Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser Thr Ala
            340                 345                 350

Tyr Met Gln Leu Ser Ser Leu Ala Ser Glu Asp Ser Ala Val Tyr Phe
        355                 360                 365

Cys Ala Arg Arg Glu Thr Thr Thr Val Gly Arg Tyr Tyr Tyr Ala Met
    370                 375                 380

Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr
385                 390                 395                 400

Thr Pro Lys Leu Gly Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ile
                405                 410                 415

Met Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Ser Ala Ser
            420                 425                 430

Ser Ser Val Ser Tyr Met Asn Trp Tyr Gln Gln Lys Ser Gly Thr Ser
        435                 440                 445

Pro Lys Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ala Ser Gly Val Pro
    450                 455                 460

Ala His Phe Arg Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
465                 470                 475                 480

Ser Gly Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp
                485                 490                 495

Ser Ser Asn Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Asn
            500                 505                 510

Arg Ala Asp Thr Ala Pro Thr Gly Ser Glu Gln Lys Leu Ile Ser Glu
        515                 520                 525

Glu Asp Leu Asn Ser His His His His His His
    530                 535
```

(2) INDICATIONS AS TO ID NO: 5:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 57 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TATATACTGC AGCTGCACCT GCGACCCTGG GCCACCAGCG GCCGCAGCAT CAGCCCG 57

(2) INDICATIONS AS TO ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTGAATTC CAGGTGCAAC TGCAGCAGTC TGGGGCTGAA CTGGC 45

(2) INDICATIONS AS TO ID NO: 7:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGTCGACGTT AACCGACAAA CAACAGATAA AACG 34

(2) INDICATIONS AS TO ID NO: 8:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 348 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: genome DNA
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) POSITION: 1..348
- (ix) FEATURE:
  - (A) NAME/KEY: mat_peptide
  - (B) POSITION: 1..348
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC       48
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
  1               5                  10                  15

GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA       96
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
             20                  25                  30

ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC      144
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
         35                  40                  45

GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG      192
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
     50                  55                  60

TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA      240
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

TCT CTC GAG AAA AGA GAG GCT GAA GCT GAA TTC CAG GTG CAA CTG CAG      288
Ser Leu Glu Lys Arg Glu Ala Glu Ala Glu Phe Gln Val Gln Leu Gln
                 85                  90                  95

CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG AAG ATG TCC      336
Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys Met Ser
            100                 105                 110

TGC AAG GCT TCT                                                      348
Cys Lys Ala Ser
        115
```

2) INDICATIONS AS TO ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) KIND: amino acid
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Glu Phe Gln Val Gln Leu Gln
85 90 95

Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys Met Ser
100 105 110

Cys Lys Ala Ser
115

(2) INDICATIONS AS TO ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: genome DNA

(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) POSITION: 1..354
(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) POSITION: 1..354
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA GCA TCC TCC     48
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA     96
Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC    144
Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45

GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG    192
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA    240
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

TCT CTC GAG AAA AGA GAG GCT GAA GCT GAA TTC ATG GCG CAG GTG CAA    288
Ser Leu Glu Lys Arg Glu Ala Glu Ala Glu Phe Met Ala Gln Val Gln
                 85                  90                  95

CTG CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG AAG    336
Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys
            100                 105                 110

ATG TCC TGC AAG GCT TCT                                            354
Met Ser Cys Lys Ala Ser
        115
```

2) INDICATIONS AS TO ID NO:11:
   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 118 amino acids
      (B) KIND: amino acid
      (D) TOPOLOGY: linear
   (ii) KIND OF MOLECULE: protein
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
 1               5                  10                  15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
            20                  25                  30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
        35                  40                  45
```

```
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
     50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                  70                  75                  80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Glu Phe Met Ala Gln Val Gln
                 85                  90                  95

Leu Gln Gln Ser Gly Ala Glu Leu Ala Arg Pro Gly Ala Ser Val Lys
            100                 105                 110

Met Ser Cys Lys Ala Ser
        115
```

(2) INDICATIONS AS TO ID NO: 12:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
TCACACAGAA TTCTTAGATC TATTAAAGAG GAGAAATTAA CC                    42
```

(2) INDICATIONS AS TO ID NO: 13:
- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 base pairs
  - (B) KIND: nucleotide
  - (C) STRAND TYPE: single strand
  - (D) TOPOLOGY: linear
- (ii) KIND OF MOLECULE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"
- (iii) HYPOTHETICAL: no
- (iv) ANTISENSE: no
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGCACACGAT ATCACCGCCA AGCTTGGGTG TTGTTTTGGC 40

(2) INDICATIONS AS TO ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"
(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

AGCACACAAG CTTGGCGGTG ATATCTTGCT CACCCAAACT CCA 43

(2) INDICATIONS AS TO ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15

AGCACACTCT AGAGACACAC AGATCTTTAG TGATGGTGAT GGTGATGTGA GTTTAGG 57

(2) INDICATIONS AS TO ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear

(ii) KIND OF MOLECULE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"
(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CAGCCGGCCA TGGCGCAGGT GCAACTGCAG CAG 33

(2) INDICATIONS AS TO ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) KIND: nucleotide
(C) STRAND TYPE: single strand
(D) TOPOLOGY: linear
(ii) KIND OF MOLECULE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"
(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TATATACTGC AGCTGCACCT GGCTACCACC ACCACCGGAG CCGCCACCAC CGCTACCACC 60

GCCGCCAGAA CCACCACCAC CAGCGGCCGC AGCATCAGCC CG 102

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

Multivalent Antibody Constructs

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on *(if applicable)*
☒ was filed in the United States as Application No. 09/674,794 *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. **PCT/DE99/01350** on **5 May 1999** and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
198 19 846.9	Germany	5 May 1998	YES ☒ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* for use only when the application is assigned to a company, partnership or other organization.

PENNIE & EDMONDS LLP DOCKET NO. 8484-089-999

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME LITTLE	FIRST NAME Melvyn	MIDDLE NAME	
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		SIGNATURE OF INVENTOR 201		DATE 9.7.01	
202	FULL NAME OF INVENTOR	LAST NAME KIPRIYANOV	FIRST NAME Sergej	MIDDLE NAME	
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		SIGNATURE OF INVENTOR 202		DATE 01.07.01	

CA1 - 259025.1